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Canada

Cellulase From The White-Rot Fungus *Polyporus anceps*

RENEWABLE ENERGY BRANCH

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Preface

This report describes work done on a project funded by Energy, Mines and Resources Canada under their Bioenergy Development Program. The purpose of the project was to compare the ability of two specific fungi to produce cellulase enzymes. Cellulases convert cellulose to sugars which in turn can be used as a substrate for fermentation. Since cellulose is a major component of biomass, cellulases will play an important role in the use of biomass as a feedstock for fermentations. Indeed, the availability of cellulase in sufficient quantity and at low cost will be essential if fermentation of biomass is to be technically and economically feasible.

One of the fungi used in this work was Trichoderma reesei RUT-C30, the product of a program at Rutgers University to select for enhanced cellulase synthesis. T. reesei RUT-C30 is currently considered to be one of the best-known producers of fungal cellulase. It was used as a yardstick against which to assess the cellulase production of Polyporus anceps, a fungus which had been found to convert a large proportion of poplar wood to sugar in previous work in this laboratory. If P. anceps could be shown to be equivalent to or better than T. reesei C30 as a cellulase producer, it would make a valuable addition to the organisms currently being considered for use in biomass conversion processes.

The work described herein was a laboratory study and would be of interest mainly to other researchers studying fungal cellulase production.

Abstract

Conversion of lignocellulosics to liquid fuels is one way of exploiting biomass as a renewable energy source. A key step in the conversion is the enzymatic hydrolysis of cellulose to sugar. To do this economically, large amounts of inexpensive and highly effective cellulase are needed. Polyporus anceps, a cellulolytic wood-degrading fungus, gave indications in some earlier work in this laboratory of an ability to hydrolyse substantial amounts of poplar shavings to sugar. The present study was undertaken to assess the cellulolytic potential of P. anceps by comparing it to Trichoderma reesei RUT-C30. T. reesei RUT-C30 is a mutant selected at Rutgers University for high cellulase yield. It is one of the best known producers of fungal cellulase and has been widely studied.

The activities of the various components of the cellulase complex were monitored in culture filtrates from submerged fermentations of the two fungi growing on purified cellulose or milled poplar. Compared to T. reesei, on neither substrate was P. anceps superior. Filtrates from both fungi showed similar activities against carboxymethylcellulose and salicin when the growth substrate was purified cellulose, but with P. anceps, the Avicel and filter paper activities were poor. When the fungi were grown on milled poplar, T. reesei filtrates showed more activity against filter paper, carboxymethylcellulose and xylan than did P. anceps filtrates. Filtrates produced by T. reesei grown on

poplar had similar filter paper, carboxymethylcellulose and xylan degrading activities to those produced by T. reesei grown on cellulose. Tests of the hydrolytic potential of culture filtrates from the two fungi supported the measurement of enzyme activities. T. reesei filtrates were superior in the release of sugars from filter paper, Solka Floc and milled poplar at all times during a 72 h incubation period.

It was concluded that when compared in submerged culture experiments, this strain of P. anceps was inferior to T. reesei (RUT-C30) in its ability to produce cellulase. As a result, P. anceps is unlikely to be a serious competitor to T. reesei without undergoing the extensive selection process which has produced this strain of T. reesei.

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Executive Summary

The possibility of converting renewable resources such as plant biomass into conventional liquid fuels has stimulated much research and development. One route for the conversion is to use microorganisms to first convert the carbohydrates in plant biomass into sugars and then ferment the sugars to alcohols. The plant carbohydrates of interest are cellulose, a polymer of glucose, and xylan, a polymer of xylose. Cellulose is the major component by weight. The biological conversion of cellulose to glucose is effected by a complex of enzymes generally referred to as cellulase. These enzymes are produced by a limited range of organisms with cellulolytic fungi being the most important in terms of industrial processes. Conversion of cellulose to glucose is presently a limiting step in technologies for making liquid fuels from biomass. The enzymatic reaction is slow and requires large amounts of expensive enzymes. As a result, one focus for research activity is to identify new sources and processes for improving yields of cellulase. The purpose of the present study was to assess the cellulase-producing abilities of the fungus Polyporus anceps (F-784-S). In previous work in this laboratory, P. anceps was found to be able to convert large quantities of poplar wood to sugar suggesting a high output of cellulase or a particularly active cellulase.

Although the original observations on P. anceps were made in solid-state culture, the present study used submerged culture

techniques as these are more common to Western industrial fermentation processes. The fungus was grown in shake flasks and fermentors on media containing either α-cellulose or milled poplar as the major growth substrate. Peptone and yeast extract were added as sources of organic nitrogen and vitamins together with a range of inorganic salts. During the fermentations samples of culture filtrate were removed at intervals and assayed for various components of the cellulase complex. These short duration (30 minutes) assays used filter paper, Avicel, carboxymethylcellulose (CMC) and salicin as substrates. Activity was determined by measuring the amount of reducing sugars released by the action of the filtrate on a given substrate. Yields of soluble protein and mycelial nitrogen were also determined. Longer duration hydrolysis experiments were conducted to test the ability of the filtrates to hydrolyse filter paper, Solka floc and poplar to sugars under more practical conditions. For comparative purposes the cellulolytic fungus Trichoderma reesei RUT-C30 was tested throughout this study under the same conditions as P. anceps. T. reesei RUT-C30, a mutant selected for cellulase yield, is currently recognized as one of the best available cellulolytic fungi.

When grown on α-cellulose in either shake flasks or fermentors, both fungi generated similar levels of enzyme activity against CMC and salicin. However, T. reesei generated much more activity against filter paper and Avicel than did P. anceps. Both fungi grew well and produced similar levels of mycelial nitrogen. They also secreted similar amounts of soluble protein into the culture filtrate. These

results suggested that P. anceps cellulase was deficient in certain components relative to T. reesei cellulase. When milled poplar was used as the growth substrate, T. reesei continued to produce high levels of activity against CMC and slightly reduced levels against filter paper. Despite the fact that P. anceps, a wood degrading fungus, finds its natural habitat on wood, it grew poorly, if at all, in this experiment and generated only minimal enzyme activities against filter paper and CMC. In the practical hydrolysis tests the rate of sugar production was high at first but quickly slowed showing the usual pattern for this type of experiment. Also, as expected, the three substrates showed increasing resistance to hydrolysis, with filter paper being the most easily hydrolysed, and milled poplar the most resistant. T. reesei filtrates always released more sugar from a given substrate than did P. anceps filtrates.

It was concluded that, under the conditions of the tests conducted, P. anceps was not able to compete successfully with T. reesei as a cellulase producer. It may be that P. anceps, a wild type strain, could produce higher yields of cellulase if subject to the same selection process as T. reesei but there would be little advantage to doing this unless the resulting strain of P. anceps had significant advantages over T. reesei. At present there is little indication that this would be so. Given that the original observations on P. anceps which led to this study were made on semi-solid cultures, it is possible that P. anceps is poorly adapted to submerged culture and might have produced more cellulase under semi-solid fermentation

conditions. However, because the semi-solid fermentation process is not commonly used in industrial fermentations in North America, this was not pursued under the present contract.

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Cellulase du champignon de la carie blanche - Polyporus anceps

Résumé à l'intention de la direction

La possibilité de convertir des ressources renouvelables, comme la biomasse végétale, en combustibles liquides traditionnels a donné lieu à de nombreuses activités de recherche et développement. L'une des façons d'effectuer cette conversion consiste à utiliser des microorganismes pour transformer d'abord les hydrates de carbone présents dans la biomasse végétale en sucres pour ensuite faire fermenter ces sucres afin d'obtenir des alcools. Les hydrates de carbone intéressants sont la cellulose, un polymère du glucose, et le xylane, un polymère du xylose. La cellulose est le constituant principal en masse. La conversion biologique de la cellulose en glucose est effectuée par un complexe d'enzymes généralement regroupées sous l'appellation cellulase. Ces enzymes sont produites par une gamme limitée d'organismes, dont les champignons cellulolytiques, qui sont les plus importants pour ce qui est des procédés industriels. Or, la conversion de la cellulose en glucose est actuellement un obstacle qui freine le développement des techniques de fabrication de combustibles liquides à partir de la biomasse. En effet, la réaction enzymatique est lente et exige des quantités importantes d'enzymes coûteuses. C'est pourquoi les recherches sont orientées entre autres vers l'identification de sources et de procédés nouveaux qui donneront de meilleurs rendements en cellulase. Le but de la présente étude était d'évaluer la capacité du champignon Polyporus anceps (F-784-S) à produire de la cellulase. Des travaux antérieurs réalisés au laboratoire avaient révélé que P. anceps pouvait transformer de grandes quantités de bois de peuplier en sucre, ce qui laissait présager que son rendement en cellulase serait élevé ou qu'il produirait une cellulase particulièrement active.

Bien que les observations originales aient été faites sur une culture de P. anceps à l'état solide, on a fait appel, pour la présente étude, à des
-2- techniques de culture submergée qui sont plus couramment utilisées dans les procédés de fermentation industriels occidentaux. Le champignon a été cultivé dans des erlenmeyers et dans des fermenteurs sur des milieux contenant ou bien de la α-cellulose ou bien du peuplier broyé comme substrat de croissance principal. On avait ajouté à ces milieux de la peptone et de l'extrait de levure comme sources d'azote organique et de vitamines ainsi qu'une variété de sels inorganiques. Pendant les fermentations, on a prélevé périodiquement des échantillons de filtrat de culture pour y rechercher divers composants du complexe cellulase. Ces essais de courte durée (30 minutes) ont été effectués avec les substrats suivants : papier filtre, Avicel, carbométhylcellulose (CMC) et salicine. On a déterminé l'activité enzymatique en mesurant la quantité de sucres réducteurs libérée par l'action du filtrat sur un substrat donné. Les rendements en protéines solubles et en azote mycélien ont également été déterminés. Par ailleurs, des expériences de plus longue durée ont été effectuées pour vérifier la capacité des filtrats à hydrolyser le papier filtre, le Solka-Floc^{md} et le peuplier en sucres dans des conditions plus pratiques. À titre de comparaison, le champignon cellulolytique Trichoderma reesei RUT-C30 a été testé tout au long de la présente étude dans les mêmes conditions que P. anceps. T. reesei RUT-C30, un mutant sélectionné pour son rendement en cellulase, est actuellement reconnu comme l'un des meilleurs champignons cellulolytiques disponibles.

Cultivés sur de la α-cellulose, que ce soit dans des erlenmeyers ou dans des fermenteurs, les deux champignons produisaient des quantités analogues d'enzyme contre la CMC et la salicine. Toutefois, l'activité enzymatique de T. reesei contre le papier filtre et l'Avicel était plus élevée que celle de P. anceps. Les deux champignons croissaient bien et produisaient des quantités analogues d'azote mycélien. Ils sécrétaient également des quantités similaires de protéines solubles dans le filtrat de culture. Ces résultats semblent indiquer que la cellulase de P. anceps était dépourvue de certains éléments présents dans la cellulase de T. reesei. Lorsque le peuplier broyé servait de substrat, T. reesei continuait à produire beaucoup d'enzyme contre la CMC et légèrement moins contre le papier filtre. Malgré le fait que l'habitat naturel de P. anceps est le bois (il s'agit d'un champignon qui dégrade le bois), ce champignon a mal poussé, sinon pas du tout, au cours de l'expérience et il n'a présenté qu'une activité enzymatique minimale contre le papier filtre et la CMC. Les essais d'hydrolyse pratiques ont révélé que la vitesse de production de sucre était élevée au début, mais qu'elle diminuait rapidement pour suivre la tendance habituellement observée dans ce type d'expérience. De plus, comme on s'y attendait, les trois substrats présentaient une résistance croissante à l'hydrolyse, le papier filtre étant le plus facilement hydrolysé et le peuplier broyé, le plus résistant. Les filtrats de T. reesei donnaient toujours davantage de sucre, à partir d'un substrat donné, que les filtrats de P. anceps.

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On a conclu que, dans les conditions des essais réalisés, P. anceps n'était pas supérieur à T. reesei en tant que producteur de cellulase. Il se peut que P. anceps, une souche de type sauvage, puisse donner des rendements plus élevés en cellulase si on la soumettait au même procédé de sélection que T. reesei.

Toutefois, il n'y a pas vraiment de raison de le faire sauf si la souche résultante de P. anceps présentait des avantages importants par rapport à T. reesei. Pour le moment, rien n'indique que ce soit le cas. Comme les observations originales sur P. anceps qui ont donné lieu à la présente étude ont été faites sur des cultures à l'état semi-solide, il est possible que P. anceps soit mal adapté à la culture submergée et qu'il aurait produit davantage de cellulase dans des conditions de fermentation à l'état semi-solide.

1. INTRODUCTION

Concern over the practical availability of finite fossil fuel resources and a desire to use other, normally non-fuel-related, resources more effectively has stimulated much research and development in the field of renewable energy resources. An important inclusion in the list of renewable resources is lignocellulosic biomass. In nature, vast quantities of lignocellulosics are degraded by the activities of a range of microorganisms which ultimately mineralize the biomass and make it available for the synthesis of new plant materials.

Among the early steps of the degradation process are delignification and the hydrolysis of carbohydrate polymers, mainly cellulose and xylan, to the sugars glucose and xylose. These sugars can be used as the starting point for the biological synthesis of alcohols, which can be used for a variety of purposes, including liquid fuels. The biological hydrolysis of cellulose, a homopolymer of glucose, is effected by the synergistic action of at least three enzymes which together are referred to as cellulase (Figure 1). Endoglucanase attacks the linkages between the glucose sub-units at random along the length of the cellulose molecule producing glucans of various chain lengths. Exoglucanase releases glucose and cellobiose, a dimer of glucose, from the reducing ends of glucans. β-glucosidase hydrolyses cellobiose to glucose. The most common producers of cellulase are microorganisms (Reese & Mandels, 1984) with fungi playing the most significant role, at least under aerobic conditions. Fungi

also are the major agents of the initial delignification of plant materials, a step which is extremely important if complete conversion of cellulose to sugars is to occur. A particularly important group of fungi here is that of the white-rot fungi which has the ability to completely degrade both lignin and cellulose (Kirk and Highley, 1973). These fungi are widely distributed in nature growing on a variety of dead or dying trees.

Research on the commercial conversion of lignocellulosics to sugars, and hence by fermentation to liquid fuels, is presently addressing a range of specific problems. Although lignocellulosics are completely degraded in nature, the process is slow by industrial standards, the concentration of the various reactants is generally low, and the overall environment is extremely complex. In addition, complete degradation is undesirable where a useful intermediate, i.e. sugar, is the required product. The most common approach, therefore, is to separate the process into discrete steps to gain more control over each one and to optimize it for industrial use: one such step is the production of cellulase (Figure 2). If processes for the conversion of lignocellulosics to liquid fuels which are to be competitive with conventional fuels are to be developed, there must be an ability to produce large amounts of cellulase. In addition to the need for large quantities, other requirements for cellulase are: low cost; high specific activity; activity against minimally treated lignocellulose; stability; and low sensitivity to feedback inhibition of sugar. Meeting these requirements remains a problem in the

development of biomass conversions based on cellulose and lignocellulosics (Reese & Mandels, 1984).

Considerable effort has gone into studies of cellulolytic fungi such as T. reesei (e.g. Montenecourt and Eveleigh, 1977; Hendy et al., 1984; Duff et al., 1987; Kawamori et al., 1986). After mutation and fermentation optimization studies, this fungus has shown remarkable increases in the yield of cellulase and is one of the best known producers of cellulase (Brown et al., 1987; Saddler et al., 1983). Nonetheless, relatively few cellulolytic fungi have been examined in detail. A case can thus be made for further screening of fungi for cellulase producers and for the examination of fungi which are found to have cellulolytic potential.

During work under contract to Agriculture Canada (DSS Contract #0SZ77-00110) (Reade & McQueen, 1983) to investigate ways of upgrading lignocellulosic materials to more digestible products for ruminant feeding, solid-state fermentations of poplar wood were conducted with five white-rot fungi, including Polyporus anceps. Of these five fungi, P. anceps was unique in the extent to which it raised the reducing sugar content of the fermenting substrate (Figure 3). Between the sixth and the eighth week of incubation, reducing sugars rose from less than 10% of the dry matter to almost 40% of dry matter. The next highest reducing sugar level, produced by P. versicolor, was only 14%. P. anceps in fact produced the most digestible product in the study, but because of the long incubation time required, when compared to the

other fungi, was not studied any further. It should be noted that the substrate used in this study was a native woody lignocellulose (poplar) which received, other than the fermentation itself, no pre-treatment to make it more digestible.

These observations suggested that this strain of P. anceps would be worth examining as a possible candidate for commercial cellulase production. The possibility existed that cellulase from P. anceps was either highly active or produced in larger quantities than cellulase from the other fungi. The relatively high reducing sugar concentration in the solid-state fermentations might also reflect an enzyme system which was less sensitive to feedback inhibition than that of the other fungi examined.

The initial proposal which resulted in the present study recommended a project in two distinct parts. In one part cellulase production was to be studied in solid-state culture because this was the source of the initial observation and the more natural growth habit of P. anceps. In the other part, the ability of P. anceps to make cellulase in the submerged fermentation systems more familiar to the Western industrial world would be examined. As a result of contract discussions with EMR the proposal was modified to include only the second of these two parts.

2. OBJECTIVES

The purpose of the study described herein was to make a laboratory comparison of cellulase production between *P. anceps* and *T. reesei* RUT-C30 and hence to determine if *P. anceps* should be used in further studies with a view to commercial cellulase production.

3. METHOD

3.1 Fungi

Polyporus anceps (F-784-S) was provided by the U.S.D.A. Forest Products Laboratory, Madison, Wisconsin, 53705. Trichoderma reesei (RUT-C30) was provided by the U.S.D.A. Agricultural Research Service Patent Culture Collection, Northern Regional Research Centre, Peoria, Illinois, 61604. P. anceps was maintained on Malt Agar (Difco) and T. reesei on Potato Dextrose Agar (Difco). Stock cultures were transferred at six-month intervals and after incubation at room temperature were stored at 4°C. Working cultures were inoculated from stocks and after 4-6 weeks incubation at room temperature were used to inoculate shake flasks.

3.2 Fermentations

Submerged cultures were carried out in 2 L shake flasks (200 mL of medium) and 7 L fermentors (5 L of medium). The medium was adapted from a recipe by Mandels and Weber (1969) and contained: KH_2PO_4 2.0 g, urea 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, peptone (Difco) 1.0 g, yeast extract (Difco) 0.5 g, Tween 80 2.0 g, $FeSO_4 \cdot 7H_2O$ 5.0 mg, $MnSO_4 \cdot H_2O$ 1.6 mg, $ZnSO_4 \cdot 7H_2O$ 1.4 mg, $CaCl_2 \cdot 6H_2O$ 2.9 mg, polypropylene glycol P2000 (antifoam) 0.5 mL, distilled water 1 L. In some fermentations as indicated below, the Tween 80 was omitted. Alpha-cellulose (Sigma) (10 g/L) was the major carbon source in most fermentations. In a few fermentations poplar wood ground in a Wiley mill to particle size of 20 mesh was used. Poplar is about 70%

carbohydrate so it was added to the fermentations at 14 g/L to completely replace α-cellulose. Some shake flask cultures used a mixture of poplar and α-cellulose (7 g/L poplar, 5 g/L α-cellulose). The shake flasks were sterilized in an autoclave at 121°C for 15 minutes. The fermentors and the 5 L batches of media were sterilized separately at 121°C for 30 minutes and the medium was then transferred aseptically to the fermentor.

Shake flasks were inoculated with spore suspensions (T. reesei) or mycelial homogenates (P. anceps) and fermentors were inoculated with 200 mL flask cultures. Spore suspensions were prepared in a solution of NaCl (10 g/L) and Tween 80 (1 g/L). Ten mL of the diluent was used for each slant (10 cm^2) of T. reesei and 1.0 mL of suspension was used to inoculate each 200 mL of medium. To prepare homogenates, two slants (20 cm^2) of P. anceps and 50 mL of diluent were blended for 30 s in a small capacity Waring blendor at high speed. Each 200 mL of medium received 5 mL of this homogenate. Shake flask cultures were incubated at 30°C on a rotary shaker at 150 rpm. When used to inoculate fermentors, the shake flasks were incubated for 3 days (T. reesei) or 5 days (P. anceps). In enzyme production experiments incubation was continued for 10-12 days. Fermentors were operated at 200 rpm stirrer speed, 2 L/min air flow and 30°C. The pH was monitored but not controlled. Samples were taken aseptically from fermentors and shake flasks at various periods as noted in the results. Shake flask and fermentor cultures were done at least in duplicate and the results reported are means of replicates.

3.3 Hydrolysis Experiment

Hydrolyses of filter paper (Whatman #1) and poplar wood both ground to 20 mesh (Wiley mill) and Solka Floc BW200 were carried out in 125 mL flasks closed with rubber stoppers. Duplicate flasks were used for each substrate. Each flask contained 5 g of substrate, 45 mL of citrate buffer (0.05 M, pH 4.8, containing 0.1 g/L sodium azide) and 5 mL of culture filtrate from shake flask cultures (*T. reesei* - 9 day cultures, *P. anceps* - 14 days cultures). The CMC activity to substrate ratio was 20 IU/g. Hydrolysis flasks were incubated in a shaking waterbath maintained at 50°C. Samples were removed at the times indicated, filtered and analyzed for reducing sugars.

3.4 Analytical Techniques

Culture samples were subdivided, and the two portions treated as follows. One portion was centrifuged at 10,000 g for 30 minutes, the sediment washed with distilled water, recentrifuged, and then freeze-dried and weighed to determine the suspended solids concentration; the dried pellet was used for mycelial nitrogen determination. The other portion was clarified by vacuum filtration on glass fibre filter paper and used for assays of enzyme activity and other soluble components of the culture. Clarified culture filtrates were stored at 4°C and analyzed within 5 days. Storage either at 4°C or at -15°C for periods of up to 44 days had a minimal effect on enzyme activity (Table 1).

Cellulase enzyme activities were measured by incubating various cellulosic substrates with diluted culture filtrate and measuring the net release of reducing sugars from the substrate (Breuil & Saddler, 1985). One mL of buffer containing substrate was mixed with 1.0 mL of culture filtrate diluted with distilled water and incubated at 50°C for 30 minutes. The enzyme reaction was stopped by adding 2.0 mL of Nelson-Somogyi (NS) sugar reagent (Nelson, 1944). A control to determine the sugar content of the unreacted mixture was prepared by adding the NS reagent immediately after the enzyme and buffer/substrate. These mixtures were not incubated. Reducing sugars (RS) were measured as described below. The substrates used were: filter paper (Whatman #1, 50 mg/tube), Avicel (FMC Corp, Type PH101, 5 mg/tube), carboxymethylcellulose (Sigma, medium viscosity, 10 mg/tube), salicin (Sigma, 5 mg/tube) and xylan (Sigma, from oat spelts, 10 mg/tube). Filter paper (FP), Avicel and xylan were suspended, and carboxymethylcellulose (CMC) and salicin were dissolved, in citrate buffer (pH 4.8, 0.05 M). Each sample of filtrate was assayed at three concentrations using duplicate reaction tubes at each concentration and the results were averaged. The concentrations were selected to yield a linear response between enzyme concentration and RS released. Enzyme activity was expressed in International Units/mL of undiluted filtrate. One International Unit (IU) corresponds to one micromole of RS produced per minute.

The RS content of enzyme assay reaction mixtures and culture filtrates was determined using Nelson's modification of the Somogyi

alkaline copper assay (Nelson, 1944). Precipitates which formed, notably with CMC, were sedimented by centrifugation at 5000 g for 10 minutes before the OD of the supernatant was measured. Glucose standard solutions (0-100 µg glucose/reaction tube) were included with each assay. These standards replaced the culture filtrate in either the enzyme assays or the straight culture filtrate RS determination.

Soluble protein in culture filtrates was measured by the Lowry method (Lorwy et al., 1951). Bovine serum albumin (Sigma) was used as a standard. Nitrogen in dried samples of suspended solids (subsequently referred to as mycelial nitrogen) was measured using Nessler's reagent (BDH) after mineralization of the samples in sulphuric acid digestion mixture (American Public Health Assocation 1981). Ammonium sulphate solution was used as a standard. All assays were done in duplicate on each sample.

4. RESULTS

4.1 Cellulase Assays

Figures 4a and 4b show the limits within which the relationship between culture filtrate assayed and amount of RS released was found to be linear for culture filtrates from T. reesei and P. anceps. If the RS concentration was allowed to exceed the highest level shown in these graphs the reaction rates declined and hence the RS vs filtrate curve became non-linear. Generally the linear range of assays of the various activities was similar for the enzymes from the two organisms. The CMC assay was the exception. With T. reesei filtrate this assay remained linear up to 320 µg RS released/tube whereas P. anceps filtrates showed marked non-linearity above 160 µg RS released/tube. It should be noted that the ordinate in the graphs of Figures 4a and 4b refers to RS released per tube. Thus, the graphs show net sugar production in the 2 mL reaction volume over any RS present in the substrate and filtrate before incubation.

4.2 Cellulase Production on α-cellulose

a) Shake Flask Cultures

Shake flask cultures containing α-cellulose showed some marked differences between the two fungi (Figures 5 and 6). P. anceps showed a 5 day lag period during which enzyme levels increased only slightly if at all and pH declined slowly. In contrast, rapid changes in enzyme levels and pH were seen early in T. reesei cultures. This may reflect the difference in the type

of inoculum used. The T. reesei inoculum consisted of asexual spores which usually germinate within 10-20 h and provide a large number of healthy mycelial growing points. The P. anceps inoculum consisted of stationary phase mycelium which had been stressed by blending. Initially, the number of growing points would have been small and the mycelium in a much less than optimal physiological state for growth and enzyme production. Once the P. anceps inoculum had adapted to the medium, the rate of protein synthesis and the accumulation of CMC and salicin activities was similar to T. reesei. Another major difference between the fungi was in the production of FP and Avicel activities. In P. anceps cultures, FP activity was low compared to T. reesei and Avicel activity was undetectable. RS concentrations remained low with both fungi. In T. reesei cultures RS concentration reached 0.06 mg/mL after 3 days and remained more or less constant thereafter. In P. anceps cultures a peak of 0.1 mg/mL was reached at 10 days followed by a slight decline to 0.09 mg/mL. It was evident from microscopic examination that cellulose substrate was disappearing as the fermentations progressed, therefore the low RS concentrations presumably resulted from the fungi rapidly metabolising the products of cellulose hydrolysis.

b) Fermentor Cultures

T. reesei was initially grown in fermentors without Tween 80 (Figures 7a and 7b) in an attempt to reduce foaming but as can be seen by comparing Figures 7a and 8a the final yield of all enzymes

was very much reduced when Tween 80 was absent. Tween 80 was therefore included in all subsequent cultures.

The initial rate of accumulation of enzyme activity with T. reesei was similar in both fermentor and shake flask culture with no apparent lag period (Figures 5 and 8a). After about 5 days, however, the initial rate had declined in shake flasks but continued up to 10 days in the fermentors. Soluble protein was almost identical in both shake flask and fermentor cultures up to 10 days (Figures 5 and 8b). As a result, the specific enzyme activities (IU/mg protein) were higher in the fermentors. RS concentrations were again low throughout the fermentation although a distinct peak occurred after 2 days. Even the highest sugar concentration (0.25 mg/mL) was very low by fermentation standards and these cultures were probably carbon limited. The suspended solids, being a measure of residual cellulose plus fungal mycelium, declined throughout the fermentation. The decline was rapid in the first 2 days and was accompanied, at first, by a rapid increase in the amount of fungal mycelium as indicated by the increase in mycelial nitrogen. This suggested a rapid conversion of cellulose to mycelium. By 2 days, however, the mycelial nitrogen had also begun a steady decline. Despite these rapid changes in synthesis and degradation of fungal biomass, the accumulation of extracellular protein and enzyme activities continued smoothly. The pH, which was controlled only by whatever buffering capacity the culture medium offered, dropped from pH 6

to pH 4.3 in the first 4 days, then climbed slowly back to 6. The initial drop may have been due to the uptake of ammonia and the subsequent climb to the hydrolysis of urea and degradation of mycelial proteins. The fluctuations in pH were not reflected by any changes in the rate of enzyme accumulation.

P. anceps fermentor cultures (Figures 9a and 9b) were inoculated with actively growing (5 day) shake cultures in contrast to the blended stationary phase mycelium used in the shake flasks. Nonetheless, there was a lag of about 1 day before enzyme production began (Figure 9a). Once enzyme production began, CMC and salicin activities accumulated rapidly until the ninth day when the production rate declined. In contrast to the P. anceps shake flask cultures, Avicel activity was at least detectable in the fermentor cultures and began to increase between 7 days and 11 days, but by this time FP activity had reached its maximum and was declining.

The various other characteristics of the fermentation showed similar trends to the T. reesei cultures, although there were quantitative differences (Figure 9b). The pH fell to about 3.7 in P. anceps cultures and had risen to only 4.5 by day 12. In the T. reesei cultures the lowest pH was 4.4 and this had risen to pH 6 by day 10. Neither enzyme activities nor protein production showed any obvious correlation with these large changes in pH. P. anceps produced approximately the same amount of extracellular

protein as T. reesei making up for the lag period by a slightly higher production rate in the ensuing 9 days. RS levels were lower with P. anceps in the early stages of the culture but gradually rose from day 2 onward.

Mycelial nitrogen concentration was similar in culture samples from both fungi whereas suspended solids remained higher in P. anceps cultures ending at 2 mg/mL compared to about 1 mg/mL with T. reesei. Suspended solids in these cultures consisted of residual cellulose plus fungal mycelium so that fungal dry weight alone could not be easily measured. The nitrogen content of washed, suspended solids was assumed to be solely the result of mycelial nitrogen and was thus an indicator of microbial biomass.

Microscopic examination of samples at the end of the culture showed little evidence of residual cellulose compared to zero time samples. It is fairly safe to assume therefore that the suspended solids at the end of the incubation periods consisted mainly of fungal mycelium. This is supported to some extent by calculation of the protein content (percent nitrogen x 6.25) of the suspended solids which gave about 20% for P. anceps cultures and about 40% for T. reesei cultures. These values are not unrealistically low for fungi, which would have been the case had the suspended solids consisted of large amounts of cellulose. One to 2 g/L, however, is a very low yield of fungal mycelium from an initial carbohydrate concentration of 10 g/L (Figures 8b and 9b). It is

likely that the yield was higher in the early phases of the culture and that considerable autolysis occurred. This was evident microscopically with T. reesei which tended to fragment and then sporulate as the cultures progressed. In P. anceps cultures, on the other hand, the mycelium seemed to remain visually intact if somewhat vacuolated. If it is assumed that the protein content of the final samples of suspended solids reflects the protein content of the mycelium throughout the fermentation, then from the mycelial nitrogen data, maximum mycelial dry weight yields of 4.5 g/L for P. anceps after 2 days and 2.6 g/L for T. reesei after 1 day can be calculated. This is, admittedly, a rather tenuous extrapolation but the figures arrived at are not unreasonable. Highly aerobic, carbon limited fungal fermentation will typically convert 40-50% of carbohydrate supplied into mycelial mass under optimal conditions. The calculated P. anceps maximum yield falls comfortably into this range. The T. reesei yield was low but was possibly influenced by the above mentioned tendency to fragment.

4.3 Cellulase Production on Milled Poplar

In this experiment the fungi were grown in flasks on media in which milled poplar was the major carbon source. Enzyme production was compared under shaken and non-shaken conditions. Additional cultures were grown in which a mixture of poplar and α-cellulose formed the growth substrate. Assays of xylanase were included because of the present of xylans in the hemicellulose of the poplar.

Table 2 summarizes the data from this experiment and Figure 10 is a graphical representation of data from the shaken cultures grown on poplar. When T. reesei grew on poplar in shaken culture, CMC and FP activities accumulated to a similar extent to when growth was on a mixture of poplar and α-cellulose (Table 2) or on α-cellulose alone (Figure 5). Under continued incubation on poplar, CMC activity continued to rise up to 21 days; however, FP activity reached a plateau at 7 days. Xylanase activity was present and showed increases throughout the 21 days incubation period. When α-cellulose was present with poplar the amount of xylanase activity was less than when poplar alone formed the carbon source. The non-shaken cultures showed poor activities of FP, CMC and xylanase. This was probably the result of poor growth due to oxygen limitation. Although it was difficult to quantitatively assess growth of the fungus on these particulate-containing media, macroscopic and microscopic examinations showed that little growth occurred in the absence of shaking whereas abundant growth occurred in the shaken cultures. P. anceps cultures generally performed poorly in this experiment (Table 2, Figure 10). Even when α-cellulose formed half the carbon source, the levels of CMC and FP activities were very low compared to T. reesei (Table 2) and to P. anceps cultures growing on α-cellulose alone (Figure 6). Microscopic examination showed little evidence of growth in any of the cultures.

4.4 Hydrolysis of Cellulosics by Culture Filtrates

Figure 11 shows the time courses of hydrolysis experiments in which T. reesei and P. anceps cultures filtrates were used to hydrolyse buffered suspensions of poplar or filter paper over a 3 day period. With the exception of the hydrolysis of poplar by P. anceps filtrate, all showed the anticipated rapid initial release of RS followed by a gradual decline in the rate of RS accumulation. P. anceps filtrate hydrolysed poplar so slowly that any initial rapid activity was hidden. T. reesei again proved to be superior and hydrolysed both substrates more rapidly than P. anceps. The importance of including practical substrates in these studies is shown by the relatively slow hydrolysis of poplar by either fungus compared to their effect on FP. Solka Floc was also tested in the present study and was found to fall between FP and poplar in its susceptibility to hydrolysis. Thus, after 72 h in the presence of Solka Floc, T. reesei filtrate had produced an RS concentration of 6.1 mg/mL and P. anceps filtrate yielded 3.6 mg/mL.

5. DISCUSSION

5.1 Cellulase Assays

Published methods for cellulase assays vary considerably in the amount of detail presented. Some methodologies which gave quantitative information (e.g. Mandels et al., 1976; Davies & Coleman, 1984) suggested that cellulolytic reaction rates would remain constant up to quite high RS concentrations (e.g. 500 µg RS/mL for CMC activity, 1 mg RS/mL for FP activity). In the present study this was not found to be the case. The data presented in Figures 4a and 4b represent the limits over which linearity was observed. Breuil and Saddler (1985) have presented data for cellulase from T. harzianum E58 in which linear reaction rates were observed over a similar range of RS concentrations to those in the present study. The difficulties in comparing data on cellulase activities between laboratories are well known (Mandels et al., 1976; Breuil & Saddler, 1985). The complex nature of the cellulase system and its insoluble substrates will likely preclude an ideal solution to these problems. A combination of practical hydrolysis tests and initial reaction velocity measurements must be coupled with an overall awareness of the limitations of these techniques when assessing cellulolytic potential. Further discussion on the topic of cellulase assays can be found in Appendix I.

The method we used to measure cellulase activity closely approaches a measurement of the initial maximum velocity of the enzyme reaction. This maximum velocity is achieved in the brief period before

product accumulation, substrate disappearance, and enzyme degradation can have an inhibitory effect on the reaction rate. While this technique offers a convenient way to compare enzyme activities it does so under very restrictive conditions. In practice cellulases will be required to effect extensive degradation of lignocellulosic substrates over relatively long periods of time. The initial reaction velocity cannot be used to predict the outcome of such an application of an enzyme. Instead, the enzyme must be tested under more practical conditions which reflect the final application. Some hydrolysis experiments of this nature are described elsewhere in this report.

5.2 Cellulase Production on α-Cellulose

It is of interest to compare absolute yields of cellulase obtained in the present study with yields reported in other laboratories. FP activity is the most frequently reported cellulase activity and several recent papers include FP activity data which may be compared with data presented herein. The highest FP activity found in the present study was 1.6 IU/mL when *T. reesei* was grown for 10 days in a fermentor. In contrast, several researchers working with the same strain of *T. reesei* have found FP activities of about 4 IU/mL (San Martin *et al.*, 1985). With one exception these results were obtained using Solka Floc at 20 g/L as the growth substrate and after 8-10 days incubation. Khan & Lamb (1984) used Whatman CF-11 cellulose at 40 g/L as their growth substrate. The lower activity found in the present study may be related to the low cellulose concentration (10 g/L) in the growth medium. FP activity in culture filtrates has been shown to increase

with cellulose concentration in the range of 0-50 g/L (San Martin et al, 1986; Hendy et al, 1984). From this, the FP activity when the cellulose growth substrate was at 10 g/L would be expected to be about 2 IU/mL which is close to the 1.6 IU/mL observed.

Saddler et al. (1985) noted about twice the activity against CMC and 1.5 times the activity against salicin compared to the present data for T. reesei. While this may again be attributed to their higher substrate concentration, this does not explain why Khan and Lamb (1984) using cellulose at 40 g/L as a growth substrate, found activity against CMC much lower, and activity against salicin similar to those in the present study. Cellulase has long been recognized as a multicomponent system (Enari & Markkanen, 1977; Wood, 1975) so that it is possible for the different components to be produced in varying amounts depending upon the precise conditions under which the fungus grows. This, together with the aforementioned analytical difficulties, could explain the above disparities.

When data from the P. anceps and T. reesei cultures are compared the major difference is seen to be the low FP and Avicel activities generated by P. anceps. Although P. anceps cultures showed a lag period before enzyme production began, the subsequent accumulation of CMC and salicin activities was actually faster and the final enzyme titre similar to the results with T. reesei. Accumulation of FP and Avicel activity, however, was much slower in P. anceps cultures with very low final titres. If maximum yields are compared, T. reesei

produced 4 times the FP activity and 9 times the Avicel activity of P. anceps. Other indicators of fungal activity such as mycelial nitrogen and extracellular protein were similar between the two fungi. In conjunction with the high level of CMC activity this suggests that the low FP and Avicel activities generated by P. anceps were not the result of generally poor growth and protein production. Under the conditions of these experiments, P. anceps was clearly inferior to T. reesei as a cellulase producer.

It might be argued that it is unfair to compare P. anceps, a wild type strain, with a highly selected, mutant such as T. reesei RUT-C30. Tables compiled by Chahal (1985) and Enari & Markkanen (1977) show the improvements in cellulase yields which have been achieved by successive selection of mutants of T. reesei QM6a, the wild type from which T. reesei RUT-C30 originated. Over a 10 year period cellulase yield has been improved about threefold. Assuming that yields from P. anceps could be similarly improved it would still be no better a cellulase producer than the mutant T. reesei.

Over 10 years ago, Enari and Markkanen (1977) noted that with hyperproducers of cellulase secreting about 2 mg/mL of protein it was unlikely that significant improvements in yield would be realized by further increases in protein synthesis. They suggested that mutants be sought which minimized the production of proteins other than cellulase and were hyperproducers of one part of the cellulase complex. By mixing filtrates from a range of such cultures a more active complete

complex might be attained. Another way of improving the hydrolytic ability of cellulase preparations has been to use mixed cultures. T. reesei cellulase is deficient in β-glucosidase activity but when T. reesei and the non cellulolytic fungus Aspergillus phoenicis are grown in mixed culture, β-glucosidase from the Aspergillus contributes to a more balanced cellulase complex with increased cellulolytic activity (Duff et al., 1986 and 1987).

Developments in fermentation techniques have also had an impact on cellulase productivity. Most noteably the fed-batch culture technique has resulted in titres of FP activity as high as 30 IU/mL (McLean & Podruzny, 1985; Hendy et al., 1984). The increased enzyme yield might result from a higher proportion of cellulase in the secreted proteins but it is likely that protein concentration in the filtrate was also significantly increased. This appears to conflict with the comments above about hyperproducers of cellulase being at the limits of their protein producing ability except that the novel approach of fed-batch culture is a more efficient way of using the fungi to convert culture medium components to cellulase than is batch culture.

5.3 Cellulase Production on Milled Poplar

In schemes for converting biomass to glucose by enzymatic hydrolysis, a major part of the price of the final product is the cost of the enzymes. In turn, the fermentation substrate is a large factor in the cost of producing the enzymes (Reese & Mandels, 1984). Substrates such as the α-cellulose used in the present study are

convenient, uniform and easily available for laboratory work but are too expensive for commercial cellulase production. Several studies have, therefore, been undertaken to assess the suitability of more practical substrates including straw (Acebal et al., 1986), bagasse (Kawamori et al., 1986a) and steam-exploded wood (San Martin et al., 1986; Khan & Lamb, 1984). Filter paper activity in filtrate from T. reesei cultures grown on steam-exploded poplar was about half that when Whatman cellulase was the substrate (Khan & Lamb, 1984). Using water-washed, steam-exploded poplar, San Martin et al. (1986), however, observed only a modest reduction in FP activity which they attributed to adsorption of cellulase onto insoluble fermentation residues. Data obtained in the present study tend to confirm this observation in that FP and CMC activities with poplar as the growth substrate were similar to those obtained with α-cellulose. It is of interest to note, however, that here the poplar had received no pretreatment except for sterilization in the autoclave.

In the present study P. anceps, despite being a white-rot fungus capable in nature of the complete degradation of lignocellulosics, generated much less cellulase activity when grown on poplar than when grown on α-cellulose. The non shaken cultures were included in this experiment because of evidence that lignin degradation by some white-rot fungi can be inhibited by agitation which disturbs the intimate contact between the fungus and its substrate (Reid et al., 1985; Kirk et al., 1978). Despite this, the low enzyme activities and lack of microscopic evidence of growth suggested that P. anceps could

not adapt to growth on wood in submerged culture. It is possible that P. anceps would perform better in a solid-state culture system which to some extent mimics its natural growth habit. Solid-state culture has also been reported to be advantageous in terms of productivity. Chahal (1985) reported an increase in cellulase produced per g of substrate (straw) of 71% when a mutant of T. reesei (QMY-1) was grown under solid-state as opposed to submerged conditions.

5.4 Hydrolysis of Cellulosics by Culture Filtrates

The use of initial reaction velocities to assess enzyme activities in culture filtrates has limitations in predicting the effectiveness of an enzyme preparation under practical conditions (Mandels et al., 1976). The assay conditions are designed to minimize the effects of changes in substrate and product concentration on reaction velocity by employing relatively short reaction times. Thus, only a small fraction of substrate is hydrolysed and correspondingly low concentrations of product accumulate. The reality of successful, practical application of cellulase requires the opposite of these conditions with extensive degradation of substrate and substantial accumulation of product. The prolonged reaction times to achieve these objectives also introduce the problem of degradation of the enzyme itself during the reaction. The net result of these factors is a steady decline in the rate of product accumulation with time rather than the linear relationship which is obtained under the special conditions of the assay for initial reaction velocity. In addition to this is the problem of attempting to apply reaction rates derived from substrates such as FP, CMC, salicin and

xylan to the more complex natural products which will most likely be used in commercial operations to obtain sugars from lignocellulosics. As a result it is useful to obtain results of more prolonged hydrolysis of cellulosic substrates in addition to the initial reaction velocity assays using specialized standard substrates which facilitate comparisons between various workers in this field.

The hydrolysis data reported herein are consistent with expectations in that the more recalcitrant substrate, poplar, was hydrolysed more slowly and to a lesser extent than filter paper or Solka Floc. The percent hydrolysis of the substrates was disappointingly low compared to previously published data. Duff et al. (1986), for example, reported about 35% conversions of Solka Floc to reducing sugar after 70 h incubation with T. reesei filtrates, which was about 6 times the conversion obtained here. Conditions with regard to substrate concentration, enzyme: substrate ratio (20 IU of CMC activity: g of substrate), buffer and incubation were the same in both experiments, although it is possible that the relative amounts of other cellulase components varied. This may also be the reason why P. anceps filtrates performed less well than T. reesei filtrates. In retrospect, it would have been better to calculate the enzyme: substrate ratios on the basis of FP rather than CMC activity as the former is a better general indicator of cellulolytic ability.

6. CONCLUSIONS

The objective of the present study was to test the potential of P. anceps (F-784-5) as a producer of cellulase for commercial application. In comparing this fungus with T. reesei (RUT-C30), currently one of the better cellulolytic fungi, it was clear that P. anceps was not able to compete successfully in producing cellulase by submerged fermentation in conventional batch cultures. It is possible that this culture method was unsuitable for an organism better adapted to growth in a semi-solid environment and that better yields would have been obtained using a different system. It is recognized, however, that submerged batch fermentation is the most widely used industrial fermentation technique, at least in the western world.

7. RECOMMENDATIONS

Research into the production and use of cellulase in the conversion of biomass to sugars and hence liquid fuels is being pursued worldwide. This enormous effort is driven by the extremely attractive goal of a continuously renewable supply of liquid fuels. In formulating research and development programs, the question is not so much whether to include this field but rather how it should be approached. The answer to this question will depend very much on the goals of the organization which is providing the funding for any given project. With regard to the present study, these goals led to an attempt to study cellulase production by P. anceps in a fermentation system to which it may have been poorly adapted. It may have been preferable to initiate the study using solid-state fermentation, the system in which the original observation of high sugar yields was made, and to move to submerged fermentation at a later date.

Given that relatively few of the possibly valuable cellulolytic microorganisms in nature have been investigated, further studies of potentially useful organisms are warranted. Whether this should be done by major screening programs or as in the present instance using a single promising fungus is debatable. Studying large numbers of organisms casts a wide net but if the fermentation conditions (e.g. pH, temperature, growth substrates, etc.) are too restrictive, potentially useful organisms may be overlooked. By restricting the numbers under investigation each organisms may be examined more closely.

In addition to continuing the search for better cellulase producing microbes, research and development in other areas is also necessary. For example, improvements in fermentation techniques, as fed-batch culture, have resulted in increases in yields of cellulase as significant as those attained by selection of hyperproducing mutants. Other areas of interest include further studies on the control of cellulase synthesis and the mode of action of cellulases with a view to increasing the efficiency of hydrolysis.

The accumulation of knowledge on cellulase and its application in the conversion of biomass to sugars has been slow and steady and there seems no reason why this trend should not continue. Much of the work will be relatively small scale laboratory research of a fairly basic nature. It may be that funding sources other than EMR's Bioenergy Development Program (BDP) are better suited to support such work. Funding of a practical biomass to liquid fuel test facility, however, might be more in line with BDP's desire to apply existing technology rather than fund basic research. This would enable the department to maintain current awareness of the technology. Individual researchers and research groups would also benefit as the facility would be able to test new developments on a practical level and would give more national focus for research and development proposals.

8. REFERENCES

Acebal, C., M.P. Castillon, P. Estrada, I. Mata, E. Costa, J Aguado, D. Romero and F. Jiminez, 1986, "Enchanced Cellulase Production from Trichoderma Reesei QM 9414 on Physically Treated Wheat Straw", Appl. Microbiol. Biotechnol., 24:218-223.

American Public Health Association, 1981, "Standard Methods for the Examination of Water and Wastewater", 15th Edition, American Public Health Association, Washington, D.C., x1vii+1134pp.

Breuil, C. and J. N. Saddler, 1985, "Comparison of the 3,5-Dinitrosalicylic Acid and Nelson-Somogyi Methods of Assaying for Reducing Sugars and Determining Cellulase Activity", Enzyme Microb. Technol., 7:327-332.

Breuil, C., P. Mayers and J. N. Saddler, 1986, "Substrate Conditions that Influence the Assays Used for Determining the β-Glucosidase Activity of Cellulolytic Microorganisms", Biotechnol. Bioeng., 28:1653-1656.

Brown, J. A., S. A. Collin and T. M. Wood, 1987, "Enhanced Enzyme Production by the Cellulolytic Fungus Penicillium Pinophilum, Mutant Strain NTG III/6", Enzyme Microb. Technol., 9:176-179.

Chahal, D. S., 1985, "Solid-State Fermentation with Trichoderma Reesei for Cellulase Production", Appl. Environ. Microbiol., 49:205-210.

Davies, J. S. and R. N. Coleman, 1984, "Fungal Cellulase Production", Report # AECV84-G1, Alberta Environmental Centre, Vegreville, Alberta, iv+95pp.

Duff, S. J. B., D. G. Cooper and O. M. Cooper, 1987, "Effect of Media Composition and Growth Conditions on Production of Cellulase and β-Glucosidase by a Mixed Fungal Fermentation", Enzyme Microb. Technol., 9:47-52.

Duff, S. J. B., D. G. Cooper and O. M. Fuller, 1986, "Evaluation of the Hydrolytic Potential of a Crude Cellulase from Mixed Cultivation of Trichoderma Reesei and Aspergillus Phoenicis", Enzyme Microb. Technol., 8:305-308.

Enari, T. M. and P. Markkanen, 1977, "Production of Cellulolytic Enzymes by Fungi", Adv. in Biochem. Eng., 5:1-25.

Hendy, N. A., C. R. Wilke and H. W. Blanch, 1984, "Enhanced Cellulase Production in Fed-Batch Culture of Trichoderma Reesei C30", Enzyme Microb. Technol., 6:73-77.

Kawamori, M., Y. Morikawa, Y. Ado and S. Takasawa, 1986a, "Production of Cellulases from Alkali-Treated Bagasse in Trichoderma Reesei", Appl. Microbiol. Biotechnol., 24:454-458.

Kawamori, M., Y. Morikawa and S. Takasawa, 1986a, "Induction and Production of Cellulases by L-Sorbose in Trichoderma Reesei", Appl. Microbiol. Biotechnol., 24:449-453.

Khan, A. W. and K. A. Lamb, 1984, "Use of Pretreated Lignocellulose for the Production of Cellulases", Biotechnol. Lett., 6:663-666.

Kirk, T. K., E. Schulz, W. J. Connors, L. F. Lorenz and J. G. Zeikus, 1978, "Influence of Culture Parameters on Lignin Metabolism by Phanerochaete Chrysosporium", Arch. Microbiol., 117:277-285.

Kirk, T. K. and T. L. Highley, 1973, "Quantitative Changes in Structural Components of Conifer Woods During Decay by White- and Brown-Rot Fungi", Phytopathology, 63:1338-1342.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr and F. J. Randall, 1951, "Protein Measurement with the Folin Phenol Reagent", J. Biol. Chem., 196:265-275.

Mandels, M. and J. Weber, 1969, "The Production of Cellulases", Adv. Chem. Ser., 95:391-414.

Mandels, M., R. Andreotti and C. Roche, 1976, "Measurements of Saccharifying Cellulase", Biotechnol. Bioeng. Symp. No. 6,21-33.

McLean, D. and M. F. Podruzny, 1985, "Further Support for Fed-Batch Production of Cellulases", Biotechnol. Lett., 7:683-688.

Montenecourt, B. S. and D. E. Eveleigh, 1977, "Preparation of Mutants of Trichoderma Reesei with Enhanced Cellulase Production", Appl. Environ. Microbiol., 34:777-782.

Nelson, N., 1944, "A Photometric Adaptation of the Somogyi Method for the Determination of Glucose", J. Biol. Chem., 153:375-380.

Reade, A. E. and R. E. McQueen, 1983, "Investigation of White-Rot Fungi for the Conversion of Poplar Into a Potential Feedstuff for Ruminants", Can. J. Microbiol., 29:457-463.

Reese, E. T. and M. Mandels, 1984, "Rolling with the Times: Production and Applications of Trichoderma Reesei Cellulase", Annu. Rep. Fermentat. Proc., 7:1-20.

Reid, I. D., E. E. Chao and P. S. S. Dawson, 1985, "Lignin Degradation of Phanerochaete Chrysosporium in Agitated Cultures", Can. J. Microbiol., 31:88-90.

Saddler, J. N., C. M. Hogan and G. Louis-Seize, 1985, "A Comparison Between the Cellulase Systems of Trichoderma Harzianum E58 and Trichoderma Reesei C30", Appl. Microbiol. Biotechnol., 22:139-145.

Saddler, J. N., E. K. C. Yu, M. Mes-Hartree, N. Levitin and H. H. Brownell, 1983, "Utilization of Enzymatically Hydrolyzed Wood Hemiculluloses by Microorganisms for Production of Liquid Fuels", Appl. Environ. Microbiol., 45:153-160.

San Martin, R., H. W. Blanch, C. R. Wilke and A. F. Sciamanna, 1986, "Production of Cellulase Enzymes and Hydrolysis of Steam-Exploded Wood", Biotechnol. Bioeng., 28:564-569.

Wood, T. M., 1975, "Properties and Mode of Action of Cellulases", Biotechnol. Bioeng. Symp. No. 5, 111-137.

APPENDICES

Appendix I
Tables and Figures

Table 1: The effect of storage conditions on the CMC activity of *T. reesei* RUT-C30 culture filtrate.[a]

Duration of storage (days)	CMC activity (IU/mL)		
	4°C	frozen[b]	frozen/thawed[c]
0	10.34	11.27	10.80
9	11.62	11.62	11.23
16	11.89	12.62	12.38
23	12.40	12.50	12.70
30	10.79	11.59	11.01
44	12.37	-	12.47

a) The filtrate was from a 3 day culture grown on the medium in Table 2.

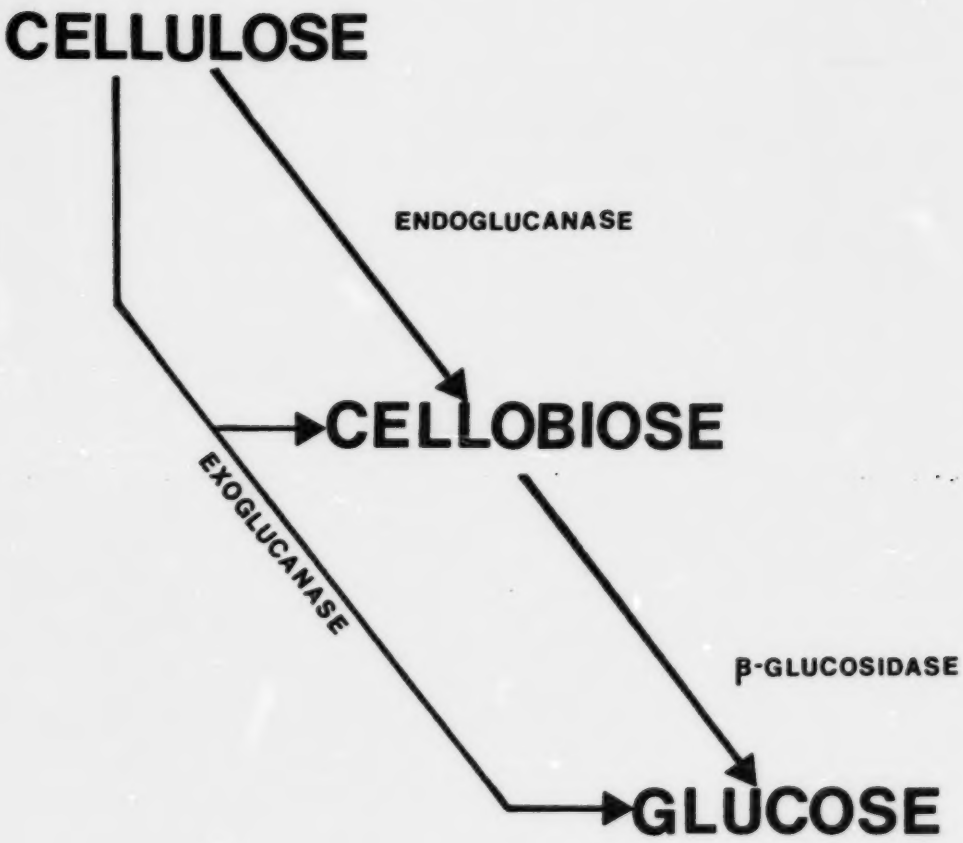
b) Small samples were kept at -15°C until analysed.

c) One sample was kept at -15°C and was thawed then refrozen on each day of analysis.

Table 2: Comparison of cellulase production[a] by *T. reesei* and *P. anceps* growing on poplar-based media in shaken or non-shaken flasks.

		POPLAR SHAKEN			POPLAR NON-SHAKEN			POPLAR/CELLULOSE SHAKEN		
Fungus	Day	FP[b]	CMC[b]	XYL[b]	FP	CMC	XYL	FP	CMC	XYL
T. REESEI	0	-[c]	3.22	-	-	2.91	-	-	3.30	-
	7	0.78	17.40	5.91	0.25	2.84	0.55	0.78	16.41	2.52
	14	0.76	26.47	5.55	0.10	4.38	0.12	0.79	23.35	2.67
	21	0.77	28.76	11.22	0.08	4.66	0.75	0.73	25.86	3.93
P. ANCEPS	0	-	1.64	-	-	0.61	-	-	1.73	-
	7	0.06	2.30	1.52	0	0.57	1.59	0.07	4.56	2.00
	14	0.02	3.19	1.01	0	0.77	0	0.03	4.83	1.28
	21	0.03	3.08	1.58	0	0.18	0.24	0	6.92	2.25

a) IU/mL
b) FP, CMC, XYL: filter paper, carboxymethylcellulose and xylan degrading activities respectively.
c) - not done



ENZYMATIC HYDROLYSIS OF CELLULOSE

Fig. 1. Enzymatic hydrolysis of cellulose.

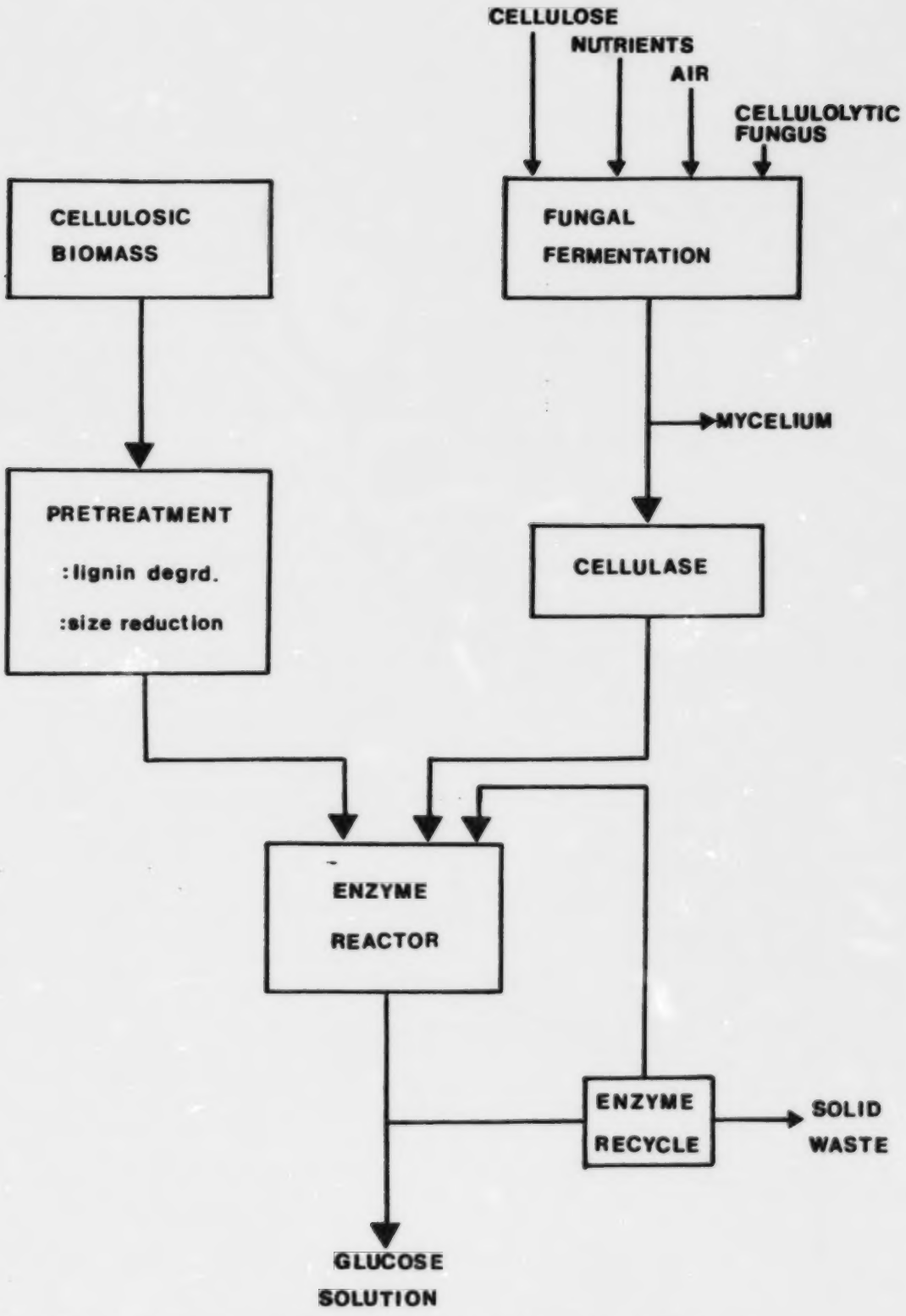


Fig. 2. Process for enzymatic conversion of cellulosic biomass to sugar.

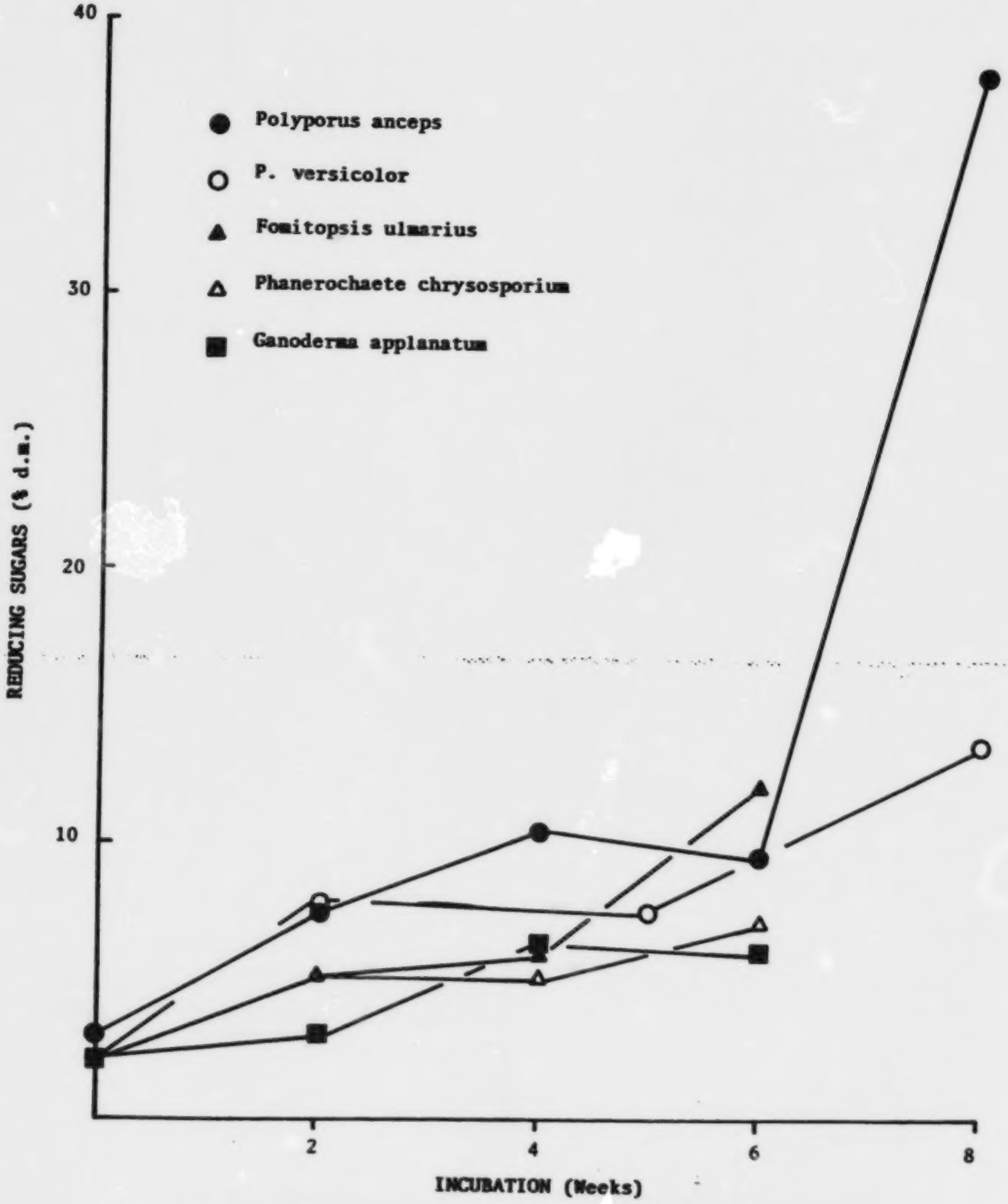


Fig. 3. Reducing sugars produced during solid state fermentation of poplar shavings by five white-rot fungi.

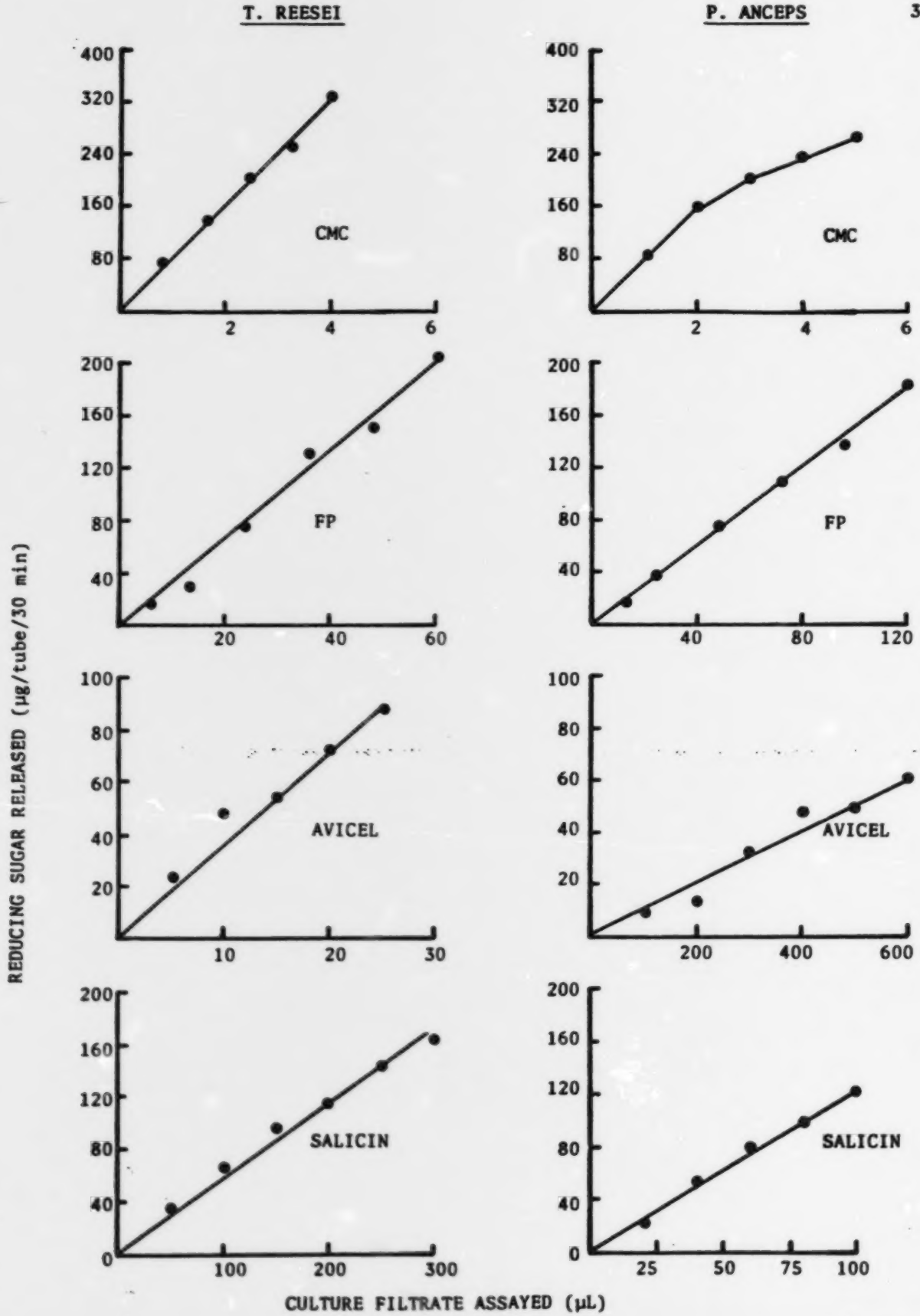


Figure 4a: Relationship between volume of culture filtrate assayed and reducing sugar released from various cellulosic substrates.

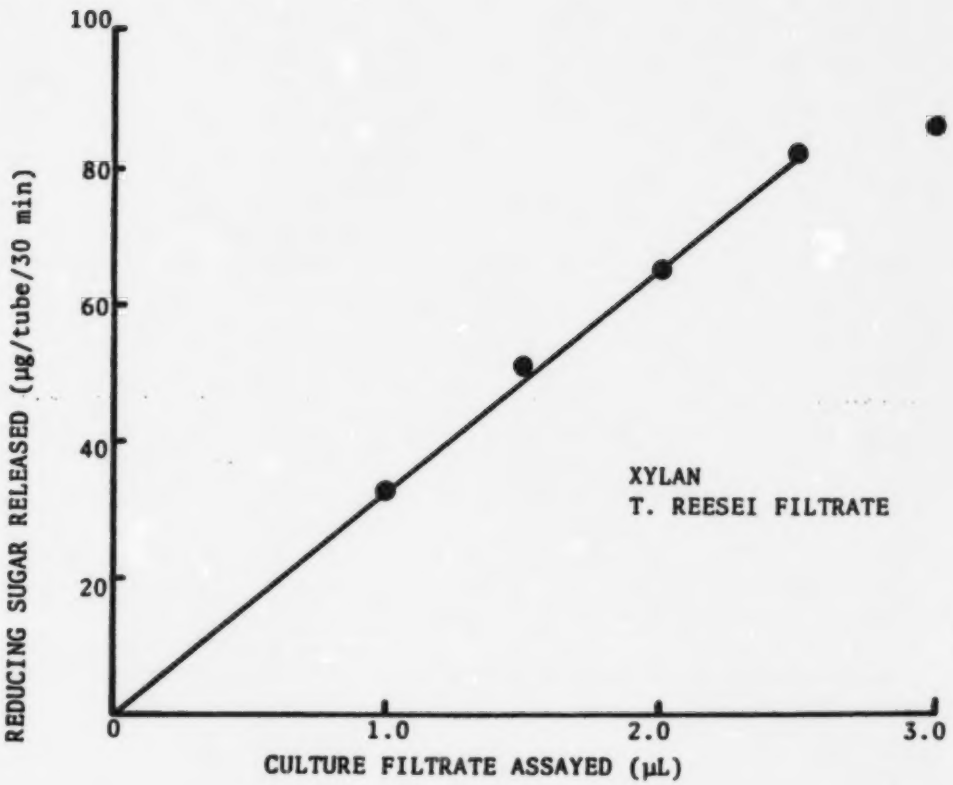


Figure 4b: Relationship between volume of culture filtrate assayed and reducing sugars released from xylan.

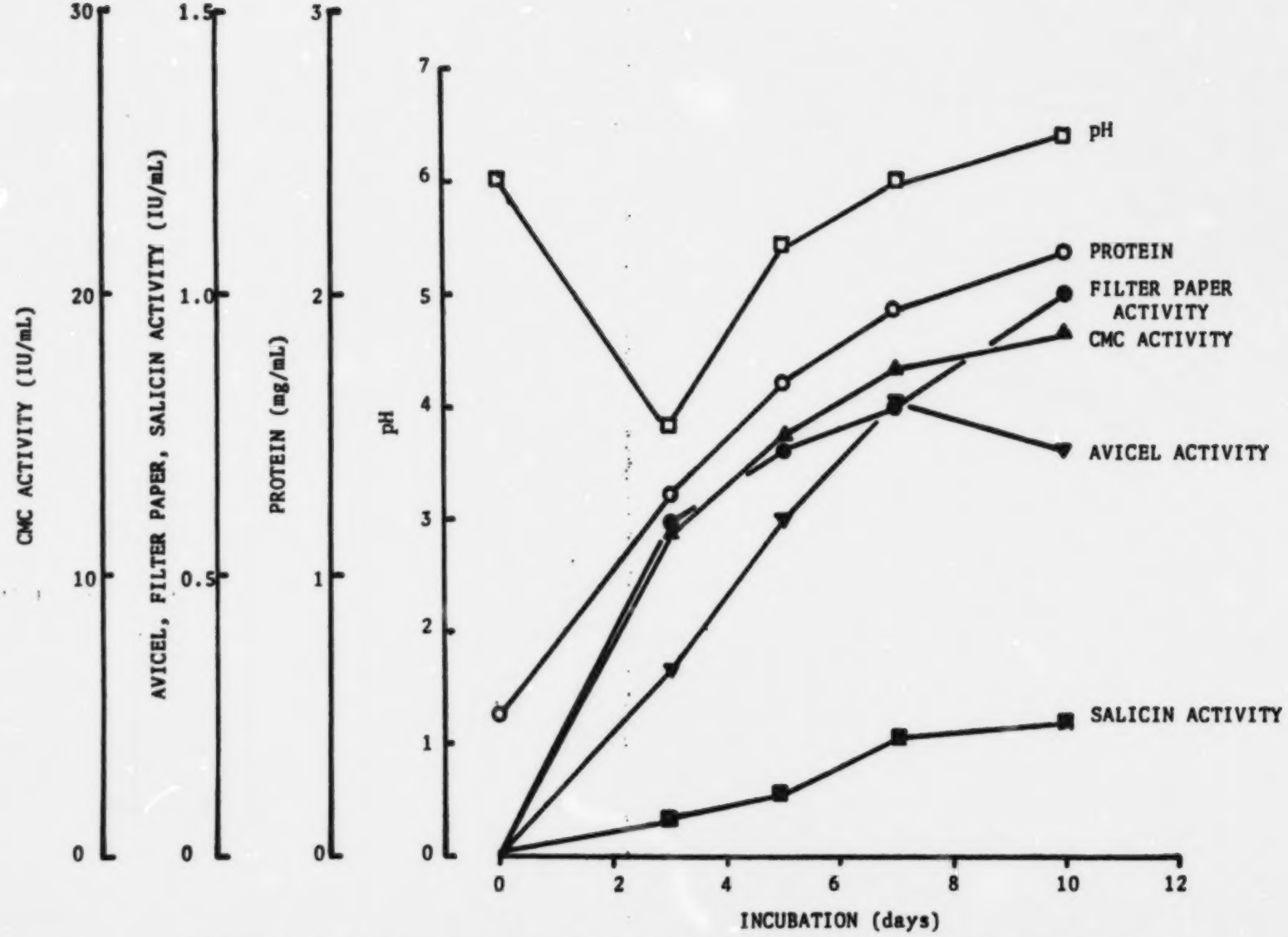


Figure 5: Cellulase production by T. reesei (RUT-C30) grown in shake flask culture

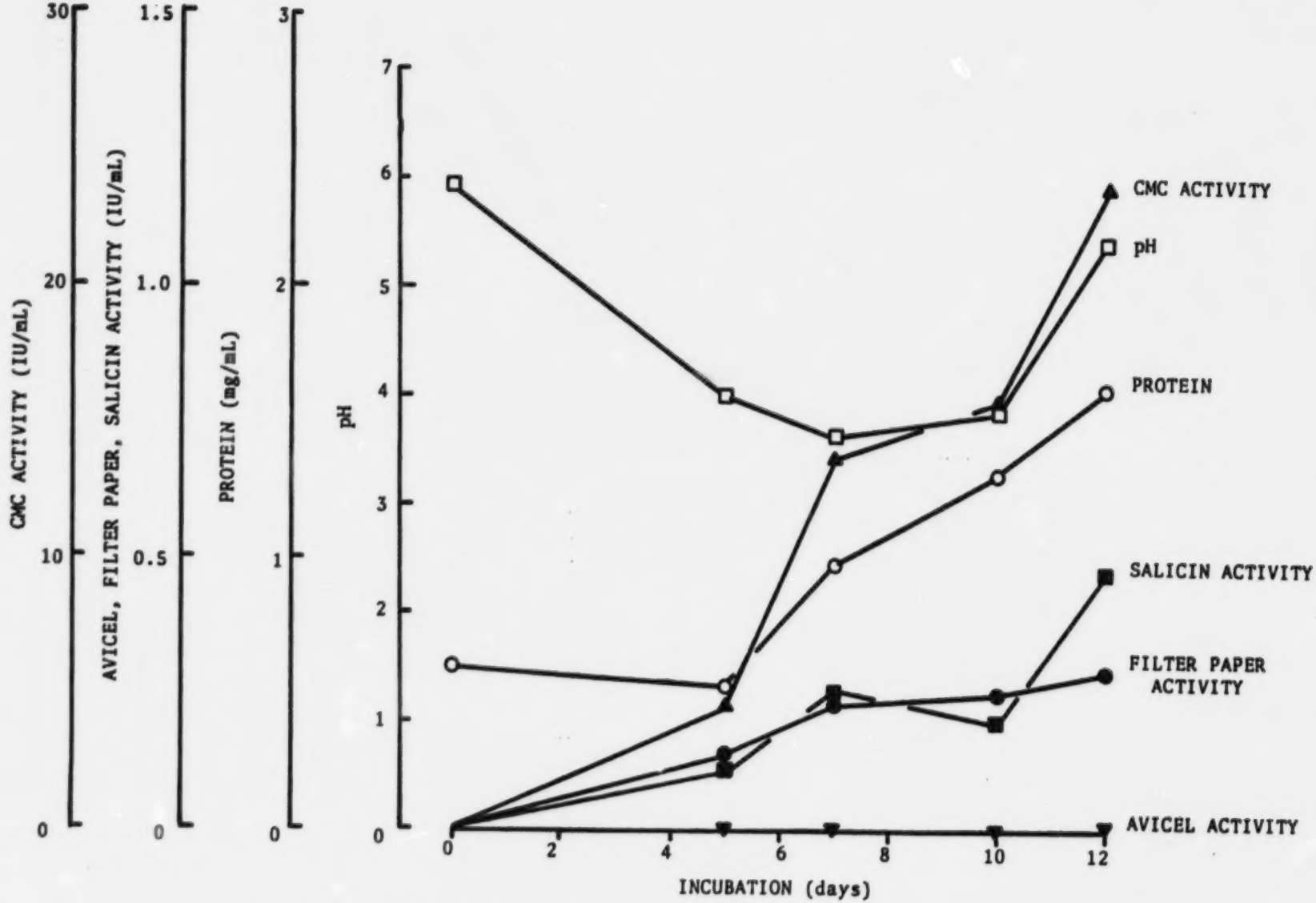


Figure 6: Cellulase production by P. anceps grown in shake flask culture

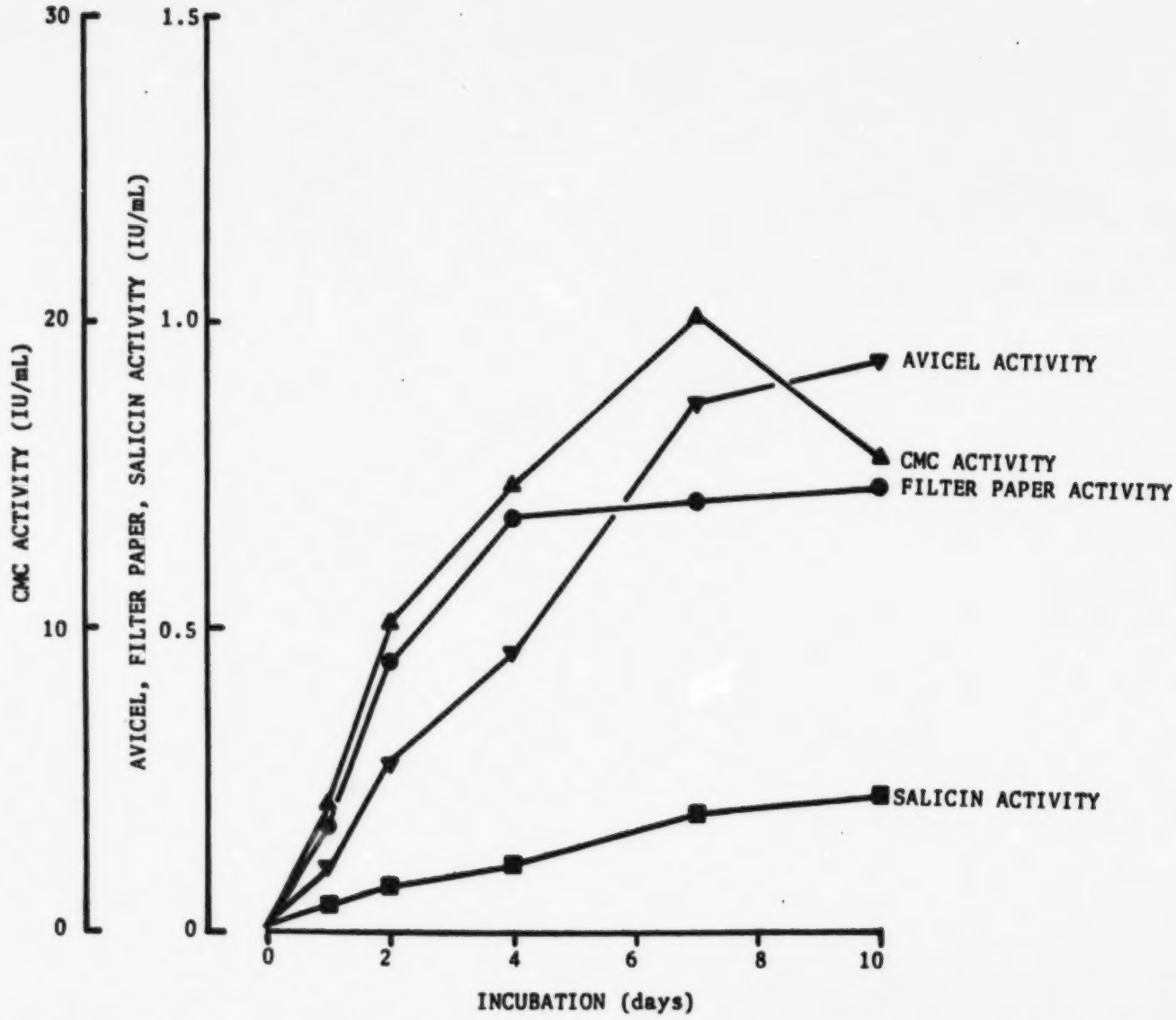


Figure 7a: Cellulase production by t. reesei (RUT-C30) in fermentor culture (medium without Tween 80).

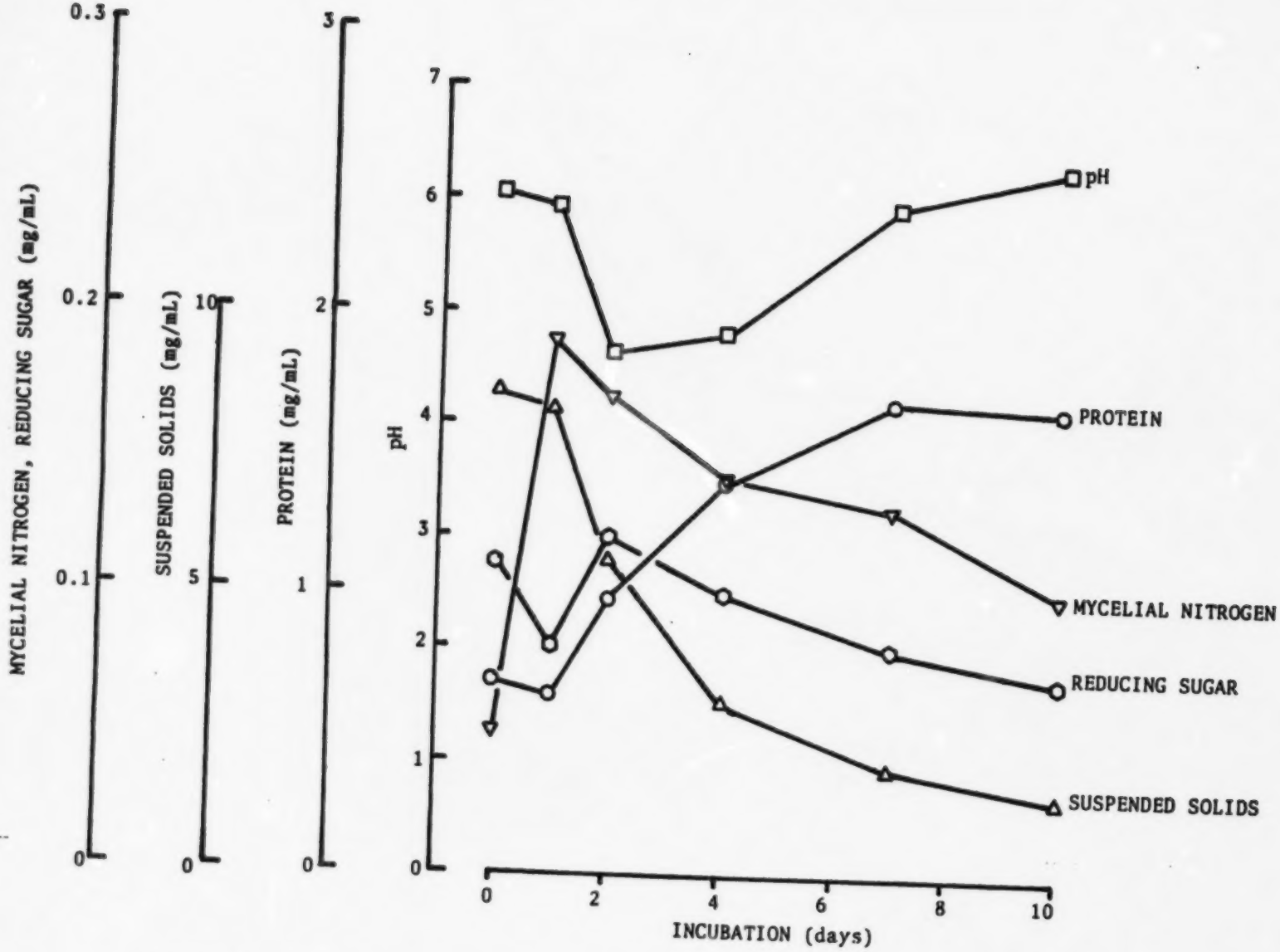


Figure 7b: Growth of T. reesei (RUT-C30) in fermentor culture (medium without T

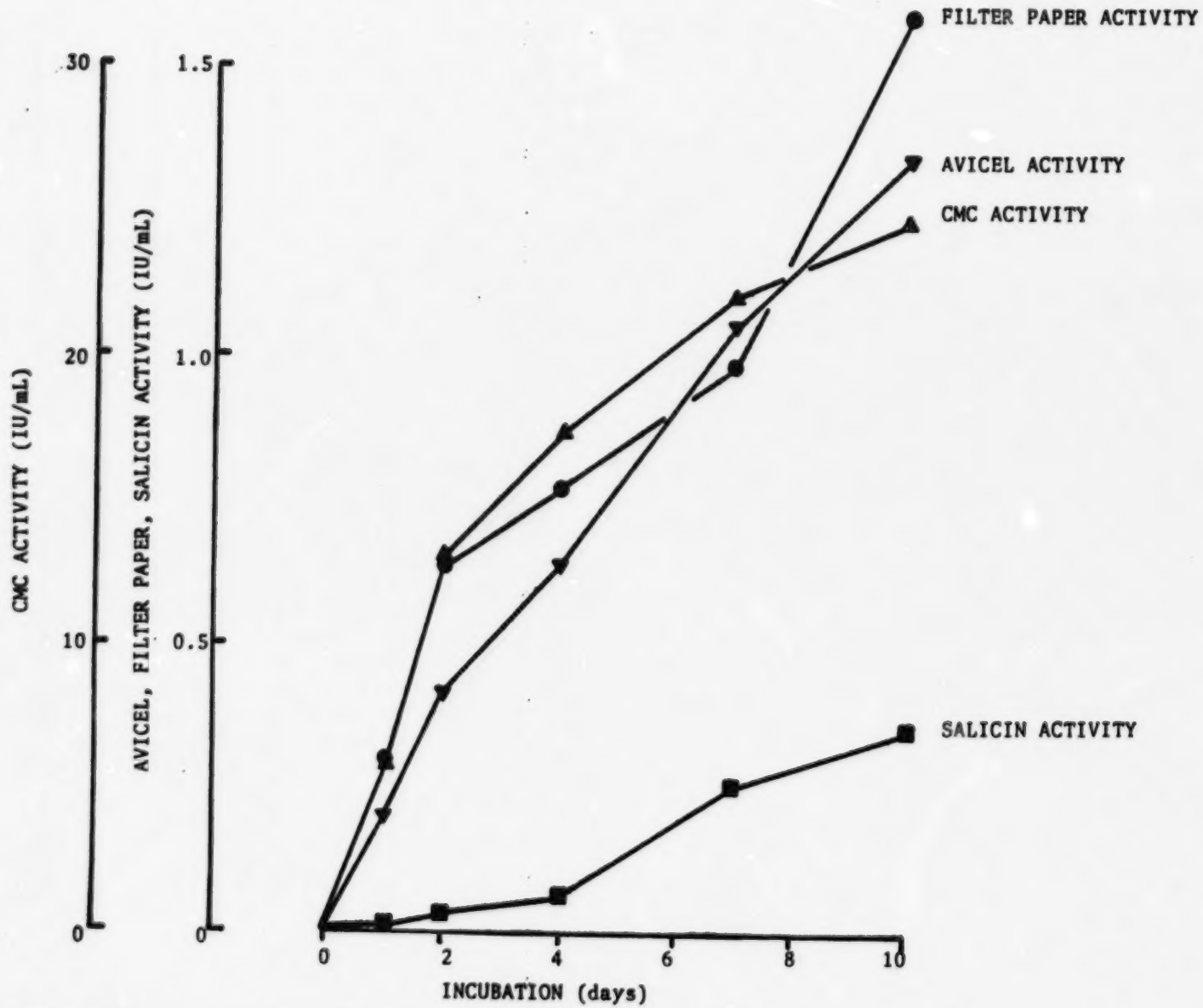


Figure 8a: Cellulase production by T. reesei (RUT-C30) in fermentor culture (medium containing Tween 80)

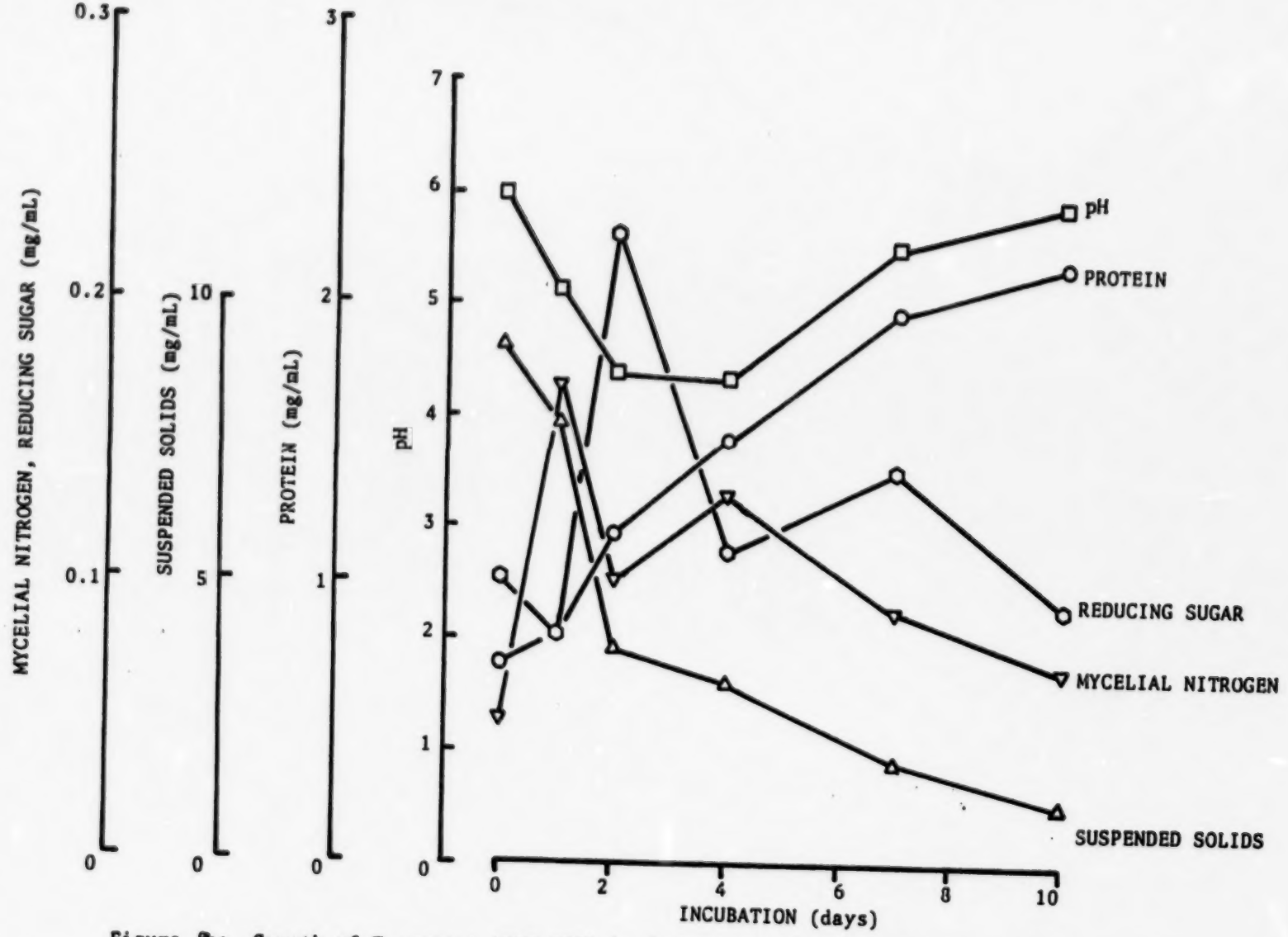


Figure 8b: Growth of T. reesei (RUT-C30) in fermentor culture (medium containing Tween 80)

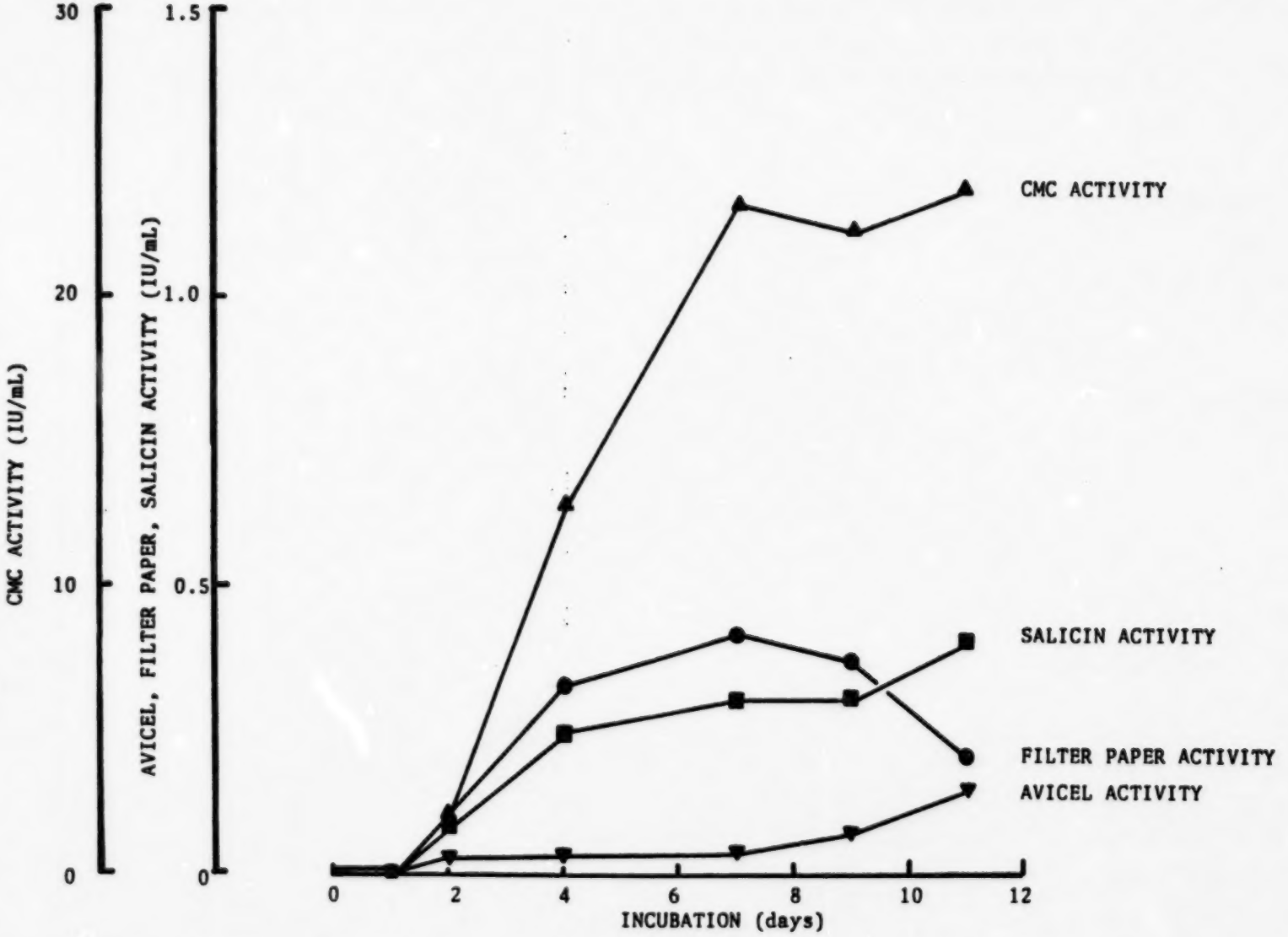


Figure 9a: Cellulase production by P. anceps in fermentor culture (medium containing Tween 80)

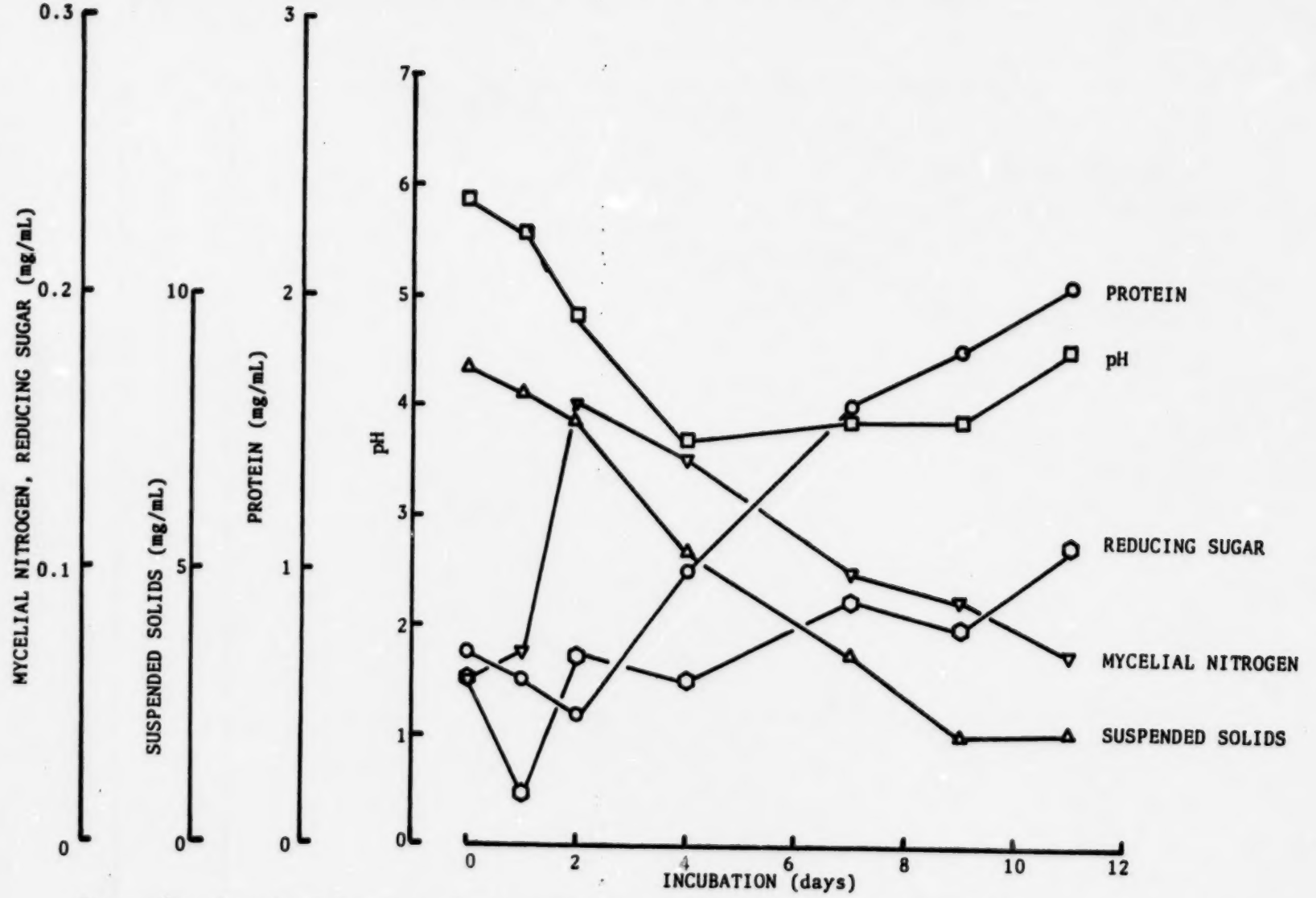


Figure 9b: Growth of P. anceps in fermentor culture (medium containing Tween 80).

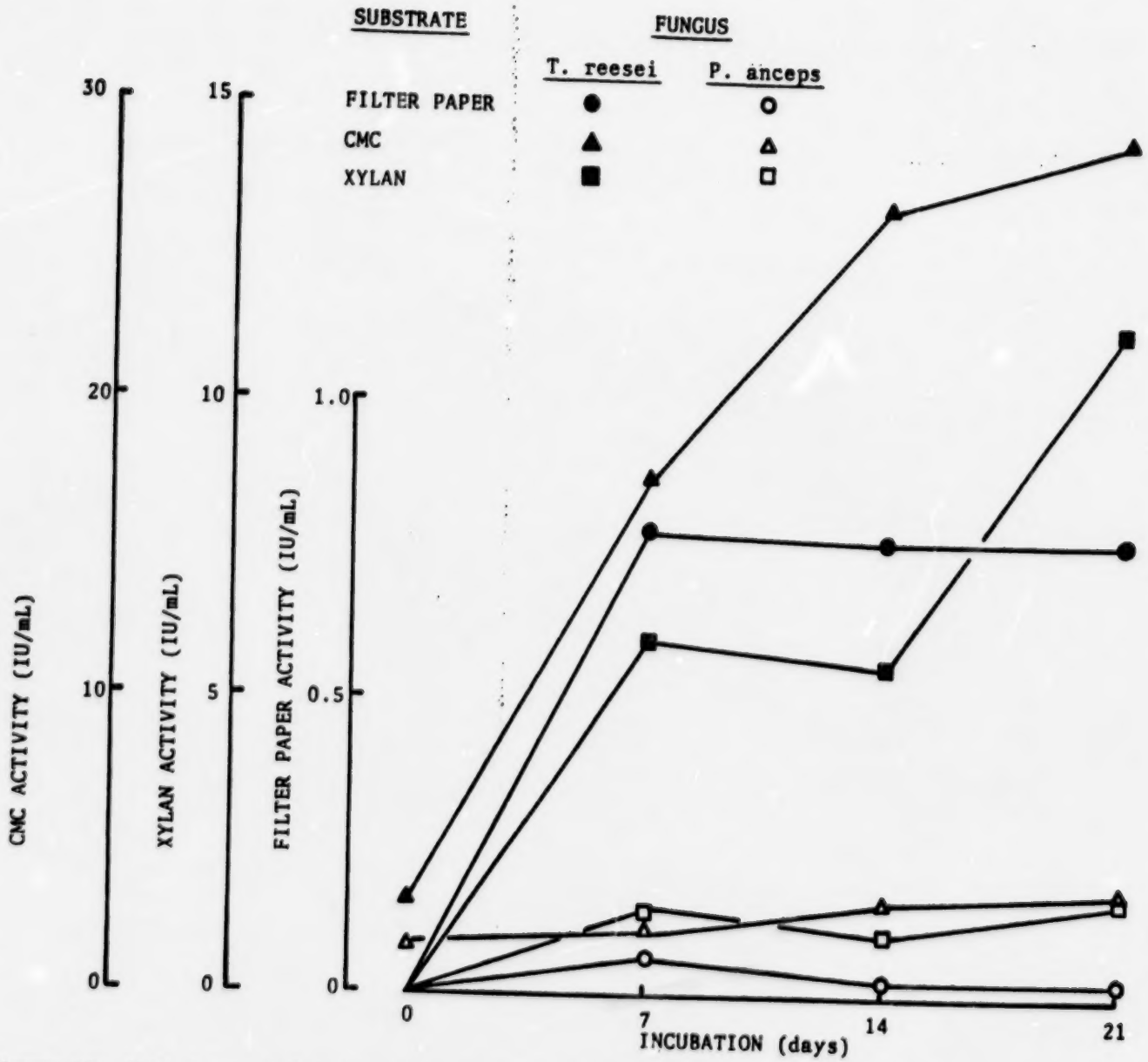


Figure 10: Cellulase production by T. reesei and P. anceps on poplar in shake flasks.

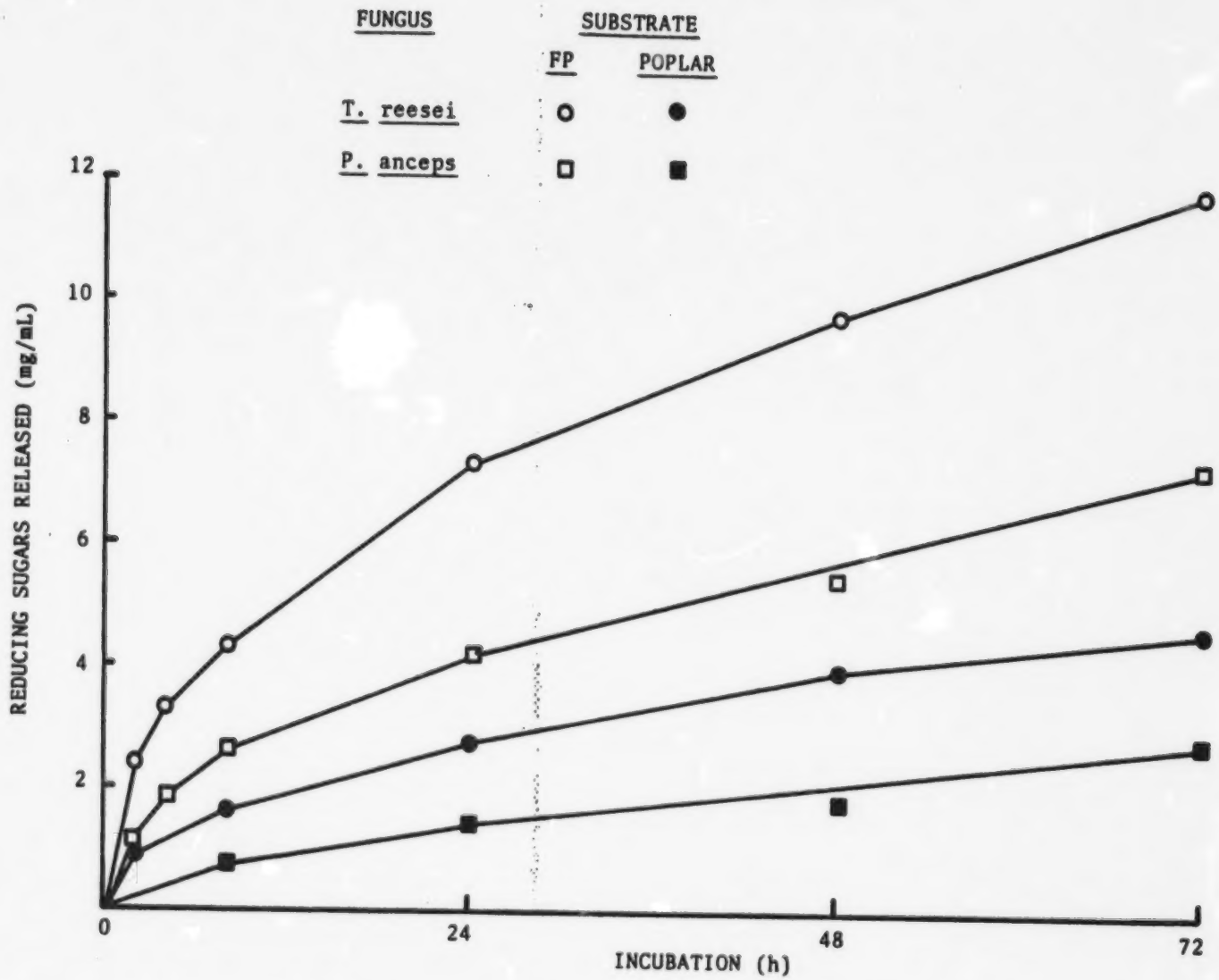


Figure 11: Hydrolysis of filter paper and poplar by culture filtrates of T. reesei and P. anceps.

Appendix II

Report on Cellulase Assay Procedures

Cellulase Assay Procedure

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INTRODUCTION

During preliminary work on a contract to compare the activity of cellulase in the culture filtrate from submerged fermentations of filamentous fungi, problems were encountered with the assay procedure selected for measuring cellulase. A number of essentially similar procedures had been found in the literature, and there was little indication of difficulties with these techniques. When we applied the methods to commercial cellulase preparations or culture filtrates prepared in our laboratory we found that the relationship between the amount of enzyme in a reaction mixture and the amount of product (reducing sugars) generated was not linear. In other words, doubling the amount of enzyme present did not double the amount of product produced. Under these circumstances it would not be possible to compare the cellulase activity of two different culture filtrates because the activity (eg. reducing sugar released per mL of filtrate per minute) would be a function of the dilution of the culture filtrate.

In order to establish an assay procedure which would allow us to proceed with the original contract, the present brief study was undertaken.

MATERIALS AND METHODS

Cellulase was produced in our laboratory by culturing Trichoderma reesei RUT-C30 which was obtained from the Northern Regional Research Centre, U.S. Department of Agriculture, Peoria Illinois. Shake flask cultures (200 mL in 2 L flasks) were incubated

at 30 C for 13 days. The culture medium for cellulase production was adapted from Mandels and Weber (1969) and consisted of: KH_2PO_4 2.0g, $(NH_4)_2SO_4$ 1.4g, urea 0.3g, $MgSO_4 \cdot 7H_2O$ 0.3g, $CaCl_2 \cdot 2H_2O$ 0.1g, peptone 1.0g, yeast extract 0.5g, Tween 80 2.0g, $FeSO_4 \cdot 7H$ 0 5.0mg, $MnSO_4 \cdot H_2O$ 1.6mg, $ZnSO_4 \cdot 7H$ 0 1.4mg, $CoCl_2 \cdot 6H$ 0 2.9mg, alpha-cellulose 10.0g, distilled water to 1L. After incubation, the mycelium was removed by filtration on Nitex cloth (100 uM aperture) and the culture filtrate was stored at 4°C. This material is referred to as C30.

A commercial cellulase preparation was also used in some tests. SP122 cellulase (a gift from Novo Enzymes) was dissolved in distilled water at a concentration of 0.5mg/mL. Further dilution was made as necessary with distilled water. These preparations are referred to as SP122.

The reducing sugar content of the enzyme reactions was measured by the dinitrosalicylic acid (DNS) method of Miller (1959) or by the alkaline copper method of Somogyi and Nelson (N-S) (Nelson 1944).

The general procedure for the enzyme assays was to incubate 1.0 mL of diluted culture filtrate with 1.0 mL of pH 4.8, 0.05M citrate buffer containing the appropriate cellulosic substrate. After incubation in a waterbath at 50°C for 30 min, the enzyme reaction was halted by adding 2.0 mL of the reducing sugar reagent. The resulting solutions were mixed and heated in a boiling waterbath

for 15 min (DNS) or 20 min (N-S) then cooled to room temperature. Six mL of Rochelle salt solution was then added to the DNS tubes and 1.0 mL of Nelson's reagent to the N-S tubes. When carbon dioxide had ceased to bubble from the N-S tubes, they were further diluted to 14 mL with distilled water. Absorbance was read in an LKB spectrophotometer, DNS tubes were read at 575 nm and the N-S tubes at 520 nm.

Exoglucanase activity was measured using filter paper as substrate. Strips of Whatman #1 paper (6 cm x 1 cm) were added to the culture filtrate/citrate buffer mixture immediately before incubation at 50°C.

Endoglucanase activity was measured using carboxymethylcellulose (CMC) as substrate. Medium viscosity CMC (Sigma) was dissolved in the citrate buffer at a concentration of 1% (w.v.).

RESULTS AND DISCUSSION

a) Reducing sugar assays

Many publications report the use of the dinitrosalycilic acid (DNS) method as modified by Miller (1959) for measuring the amount of reducing sugars in assays for cellulase (eg. Mandels *et al.* 1976, Khan & Lamb 1984). Breuil and Saddler (1985) compared the DNS reducing sugar assay with the Nelson-Somogyi (N-S) procedure (Nelson 1944) and found a number of reasons to prefer the N-S method for cellulase assays. Reducing sugar values obtained by the DNS

method were higher than those obtained with the N-S method but the DNS method was more variable. In contrast, the N-S method was not affected by components of the buffers, media or lignocellulosic materials often present in crude enzyme filtrates. Another inconvenience with the DNS method is the destruction of glucose by oxidation (Miller 1959, Breuil & Saddler 1984) which causes the standard curve to have an intercept on the x (i.e. mg of sugar) axis rather than pass through the origin. The modified DNS reagent of Miller (1959) was intended to eliminate this by the addition of an oxygen scavenger, sodium sulfite, to the reagent. In our experience, both the single part DNS reagent proposed by Sumner and Sisler (1944) and the modified two-part reagent of Miller (1959) suffered from glucose loss (Fig. 1). Increasing the sodium sulfite concentrate from 0.05% to 0.1 or 0.2% resolved this problem. In our work, however, there remained the disadvantage of the relatively low sensitivity of the DNS method. The N-S method, in addition to giving a standard curve passing through the origin, is more sensitive than the DNS method (Fig. 2). This turned out to be important as we found that the key to running a successful endoglucanase assay was to work at reducing sugar levels of less than 100 µg/mL.

Another serious drawback with the DNS method is the dependence of colour yield on the degree of polymerization of the reducing sugar (Breuil & Saddler 1984; Robyt & Whelan 1972). Even a purified exocellulase will produce various sized fragments of cellulose as the hydrolysis proceeds. A crude cellulolytic culture filtrate would likely produce an even more heterogenous product mix.

Clearly a reducing sugar assay which is sensitive to the degree of polymerization of the reducing sugar is not desirable.

b) Cellulase assays

Different enzyme preparations may be compared by reference to their activity. In the case of cellulases, this means the rate at which the enzymes hydrolyse the glycosidic bonds between the glucase sub-units of cellulose. As these bonds are hydrolysed, reducing end groups are exposed which can be measured by techniques such as the aforementioned DNS and N-S reducing sugar assays. Using glucose as a standard to calibrate the DNS or N-S assays, one can then express the activity of the enzyme preparation in International Units (IU) which are μmoles of reducing sugar released/minute (per mL or mg of the preparation being analysed). A difficulty in measuring enzyme activities exists because as an enzyme reaction proceeds, the rate of product formation may change. Typically the rate declines and this may be caused by a number of factors, eg. limited availability of substrate; inhibition of the enzyme by reaction products; a change in the reaction conditions caused by product accumulation. Provided that adequate amounts of substrate are present, it is usually possible to limit the quantity of product produced so that the reaction conditions do not change and product inhibition in susceptible enzymes is negligible. Under these circumstances the product of the enzyme reaction accumulates in a linear manner with respect to time and therefore at any given time the amount of product is directly proportional to the amount of enzyme activity.

An initial review of the literature suggested that the hydrolysis of a 1% solution of CMC, a means of measuring endoglucanase activity, should be linear until about 0.5 mg of reducing sugar had accumulated per mL of reaction mixture (Mandels et al. 1976, Davies & Coleman 1984, Saddler et al. 1985). However, when a commercial enzyme preparation (Novo SP122) and filtrates from cultures of T. reesei C30 were assayed, even though sugar production was in the 0-0.6 mg/mL range, progressively less sugar was released for unit increases in enzyme concentration (Figs. 3 & 4). This indicates that under these assay conditions the reactions were not proceeding in a linear manner. Further dilution of the enzyme was indicated and necessitated a switch to the N-S reagent in order to be able to detect the smaller amounts of sugar released. Figures 5 & 6 show that the reaction became linear when the amount of culture filtrate added was between 0 and 2 µL and the sugar released did not exceed about 150 µg/mL. The results reported by Breuil and Saddler (1985) also indicate that the assay for the endoglucanase activity of the cellulase complex is very sensitive to the amount of glucose which accumulates. This sensitivity may, of course, reflect a property of the specific enzyme being assayed. If this is the case then it is quite possible that enzymes from different species and strains of cellulolytic fungi would exhibit different degrees of sensitivity to accumulated sugar. Enzymes less affected by product accumulation would sustain linear reaction rates until quite high levels of sugar had accumulated and would account for reports of linearity up to 0.5 mg of reducing sugar/mL. Twenty-two assays of the endoglucanase activity of C30 culture filtrate done on four separate occassions gave an activity of 15.13 ± 0.88 IU/mL of filtrate.

In Breuil and Saddler's (1985) work, exoglucanase activity (filter paper substrate) seemed less susceptible to the effects of reducing sugar accumulation than endoglucanase activity. We did not try to operate the exoglucanase assay at high reducing sugar levels as the N-S reducing sugar assay permitted us to obtain satisfactory enzyme vs product lines at reducing sugar levels of 200 µg/mL or lower (Fig. 7). Assays of exoglucanase activity of the C30 filtrate gave activities of 0.76 ± 0.1 IU/mL.

By following recommendations from a number of sources, but in particular Forintek Canada Corporation staff in Ottawa, we have revised our assays for cellulase so that they now function according to classical enzyme theory. Although these assays now offer a reproducible means of assessing the enzyme content of culture filtrates using the initial, maximum reaction velocity, it must be recognized that this technique has severe limitations in predicting the effectiveness of cellulases in the practical hydrolysis of cellulose. Under practical conditions the concentration of the enzymes, substrates and products will be much higher than in the present assays and classical enzyme assay kinetics will not be applicable. A methodology which enables the researcher to conveniently compare cellulase preparations on a basis more related to their end use is not yet available.

LITERATURE CITED

Breuil, C. and J.N. Saddler. 1985. Enzyme and Microbiol. Technol. 7: 327-332.

Davies, J.S. and R.N. Coleman. 1984. Research report AECV84-G1, Alberta Environmental Centre.

Khan, A.W. and K.A. Lamb. 1984. Biotechnology Letters 6: 663-666.

Mandels, M. and J. Weber. 1969. Adv. in Chem. Series 95: 391.

Mandels, M., R. Andreotti and C. Roche. 1976. Biotechnol. Bioeng. Symp. 6: 21-34.

Miller, G.L. 1959. Anal. Chem. 31: 426-428.

Nelson, N. 1944. J. Biol. Chem. 153: 375-380.

Robyt, J.F. and W.J. Whelan. 1972. Anal. Biochem. 45: 510-516.

Saddler, J.N., C.M. Hogan and G. Louis-Seize. 1985. Appl. Microbiol. Biotechnol. 22: 139-145.

Sumner, J.B. and E.B. Sisler. 1944. Arch. Biochem. 4: 333.

FIGURE 1: Dinitrosalycilic acid method for reducing sugars

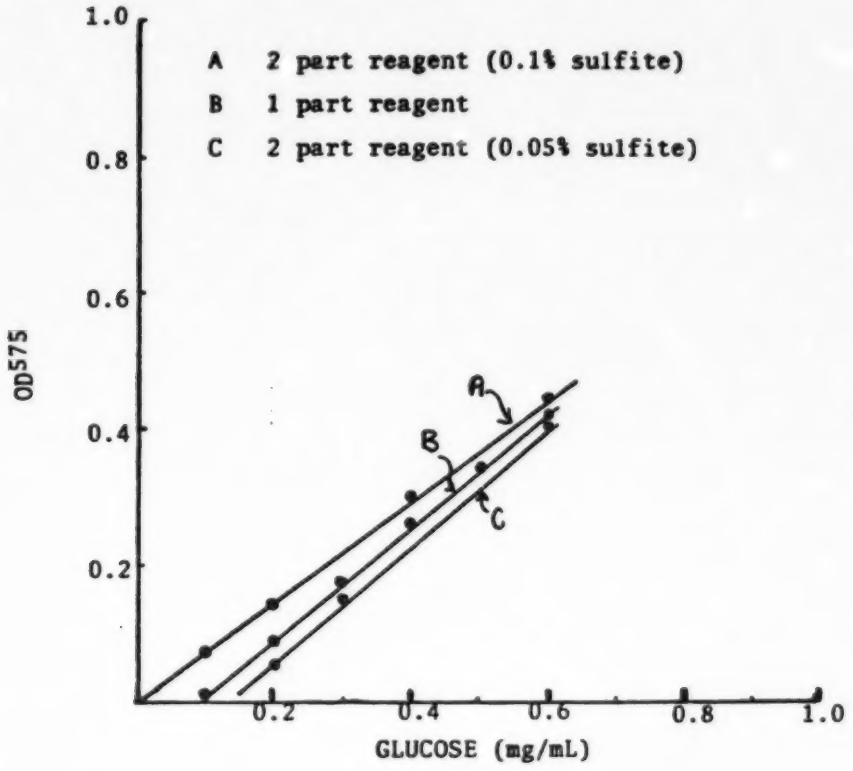

FIGURE 2: Nelson-Somogyi method for reducing sugars

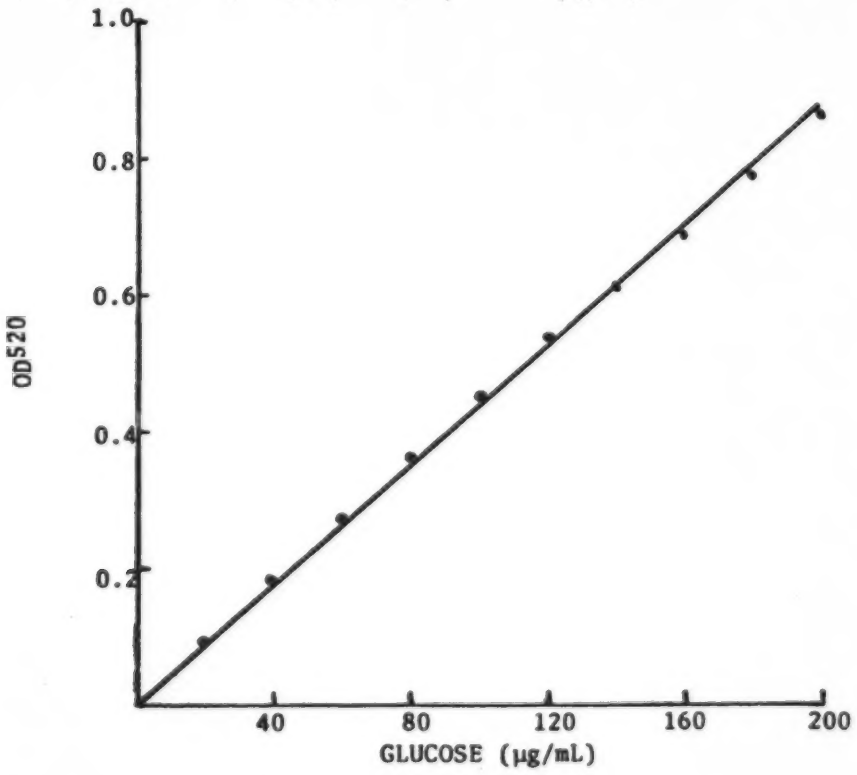

FIGURE 3: Endoglucanase activity of Novo SP122 enzyme using DNS reducing sugar assay

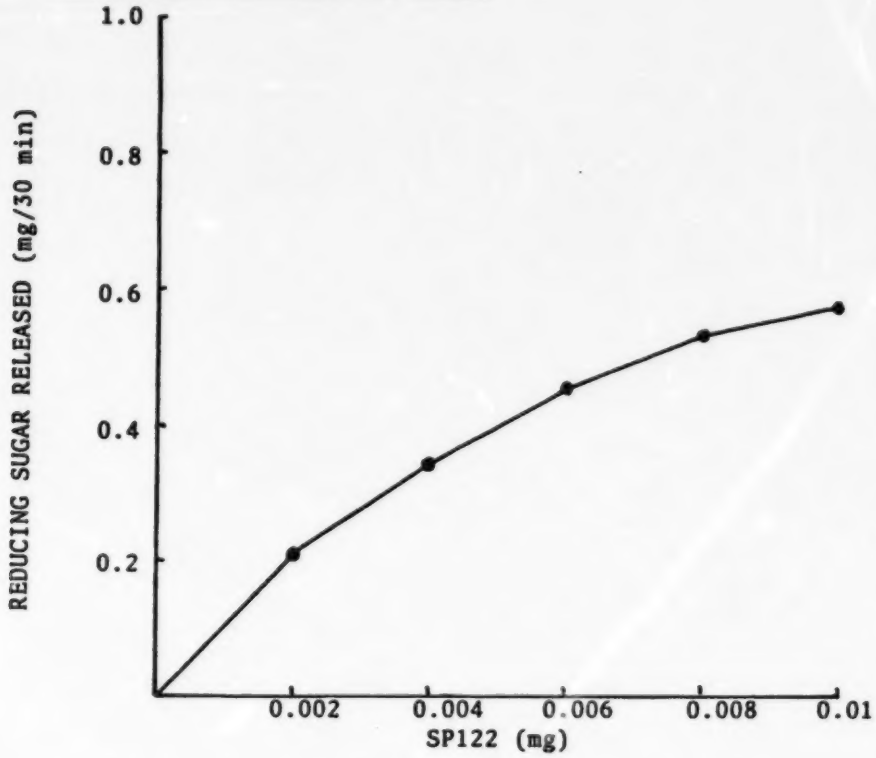

FIGURE 4: Endoglucanase activity of T. reesei RUT-30 culture filtrate using DNS reducing sugar assay

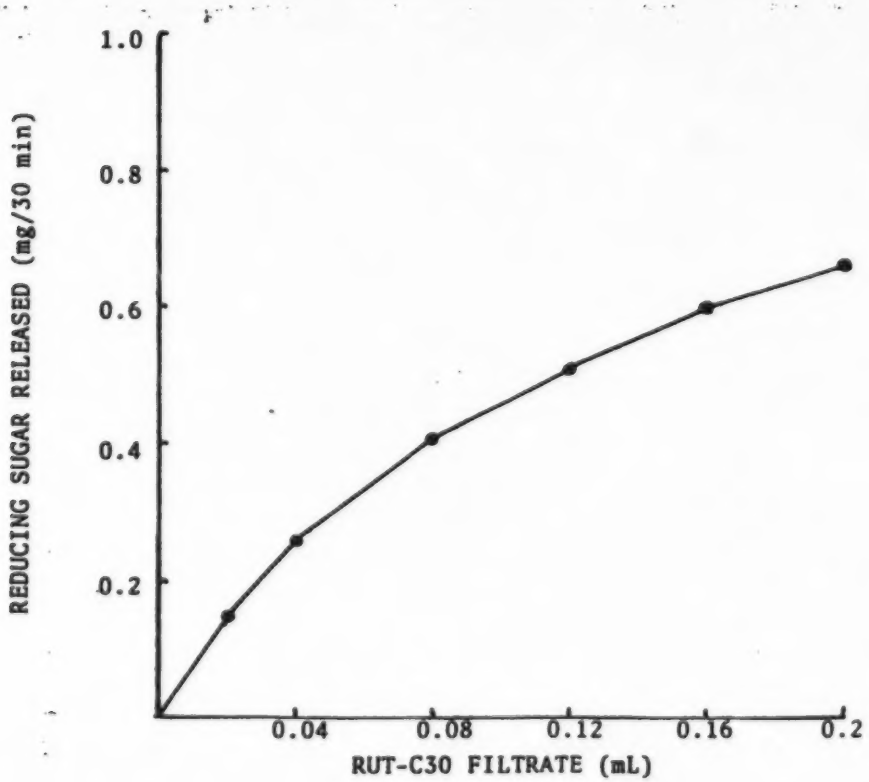

FIGURE 5: Endoglucanase activity of T. reesei RUT-C30 culture filtrate using N-S reducing sugar assay

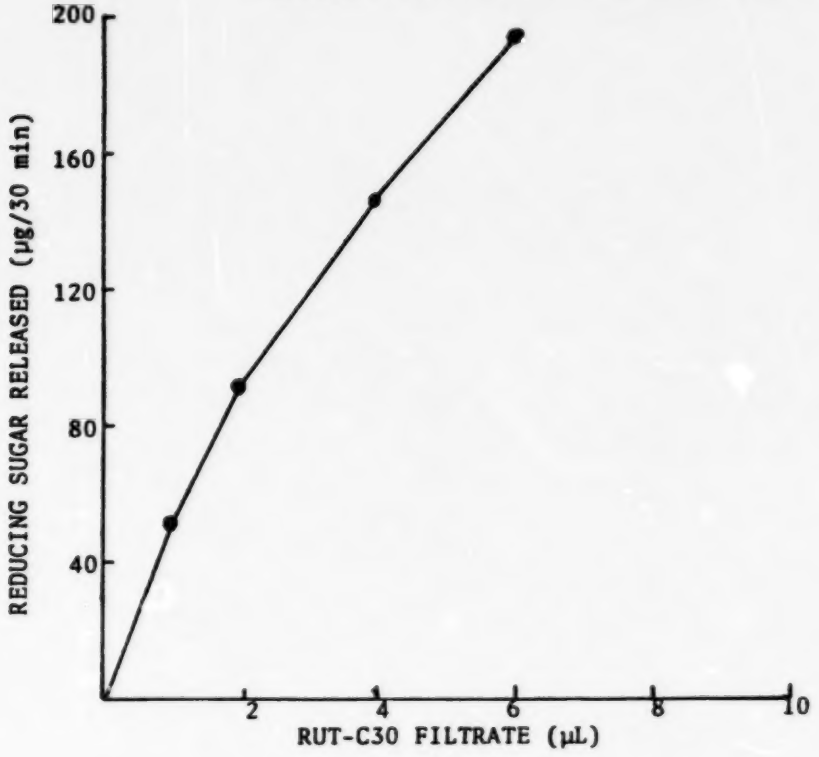

FIGURE 6: Endoglucanase activity of T. reesei RUT-C30 culture filtrate using N-S reducing sugar assay

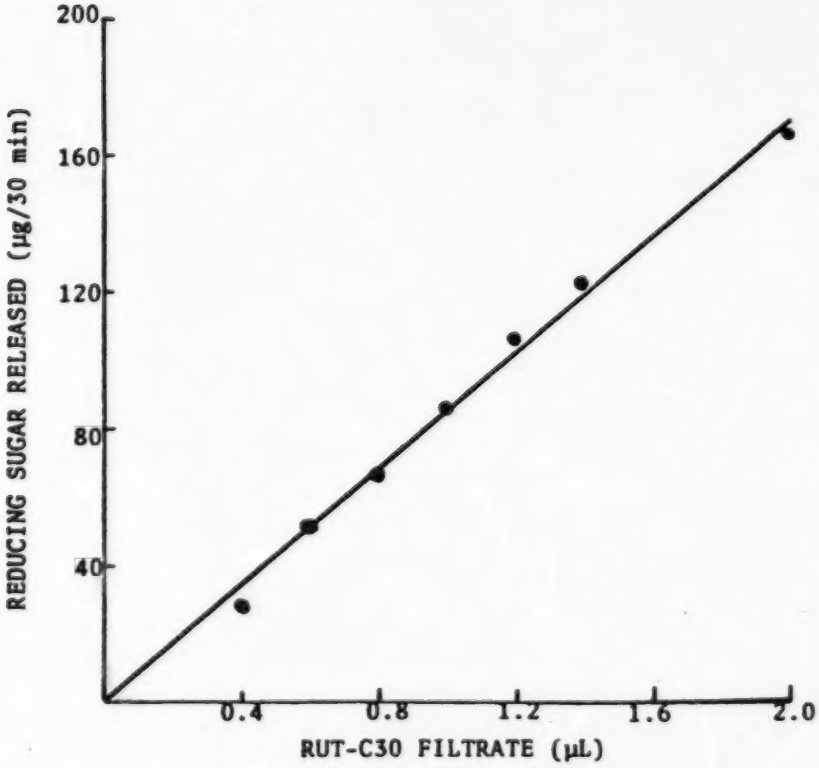

FIGURE 7: Filter paper hydrolysing activity of T. reesei RUT-C30 culture filtrate using the N-S reducing sugar assay